ROLES OF ATP BINDING CASSETTE TRANSPORTERS IN DRUG SENSITIVITY AND PHYSIOLOGY OF *PLASMODIUM* PARASITES

Proefschrift

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General Introduction

Adapted from:

The ABCs of multidrug resistance in malaria

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MALARIA

Malaria is one of the major global health problems that was estimated to cause 207 million new cases and 584.000 casualties in 2014 mainly in children under five in (sub)tropical regions **(Figure 1A)**[1]. These mortality figures are almost exclusively caused by infections with the most virulent *Plasmodium* parasite; *Plasmodium falciparum (P. falciparum).* This versatile parasite is injected into the skin of soon-to-be patients through the bite of an infected female *Anopheles* mosquito, after which sporozoites travel through the bloodstream, and infect liver cells. The liver stage of *P. falciparum* parasites lasts for approximately seven days, during which intracellular multiplication generates presumably tens of thousands of merozoites. Bursting of liver cells releases these parasites into the blood stream, where they can infect red blood cells. During the red blood cell cycle, newly invaded parasites form the typical ring stages that can be observed in the peripheral blood, after which they develop into trophozoites and schizonts, that are sequestered in microvasculature of various organs[2]. Fully matured schizonts mostly consist of 14-20 merozoites[3], that upon rupturing of the infected red blood cell infect new cells. This is the stage of the *P. falciparum* life cycle that causes the well-known malaria symptoms; intermittent high fever, muscle ache, headache, vomiting. Not only the steep reduction in red blood cells, which leads to low hematocrite values, but also the tendency of infected red blood cells to adhere to endothelium of capillaries, and thereby blocking hem, contributes to the severity of the disease[4]. In case of cerebral malaria, when oxygen supply to the brain critically inhibited, surviving patients may suffer from permanent cognitive impairment[5]. During the blood stage cycle, a small proportion of the parasites switch to gametocyte formation. Gametocytes, both male and female, can be taken up by a mosquito, and during a short diploid stage in which genomic exchange is realized, these gametes form ookinetes that traverse the mosquito midgut wall, where the oocysts mature, and after approximately 14 days, sporozoites can be detected in the mosquito salivary glands **(Figure 1B)**.

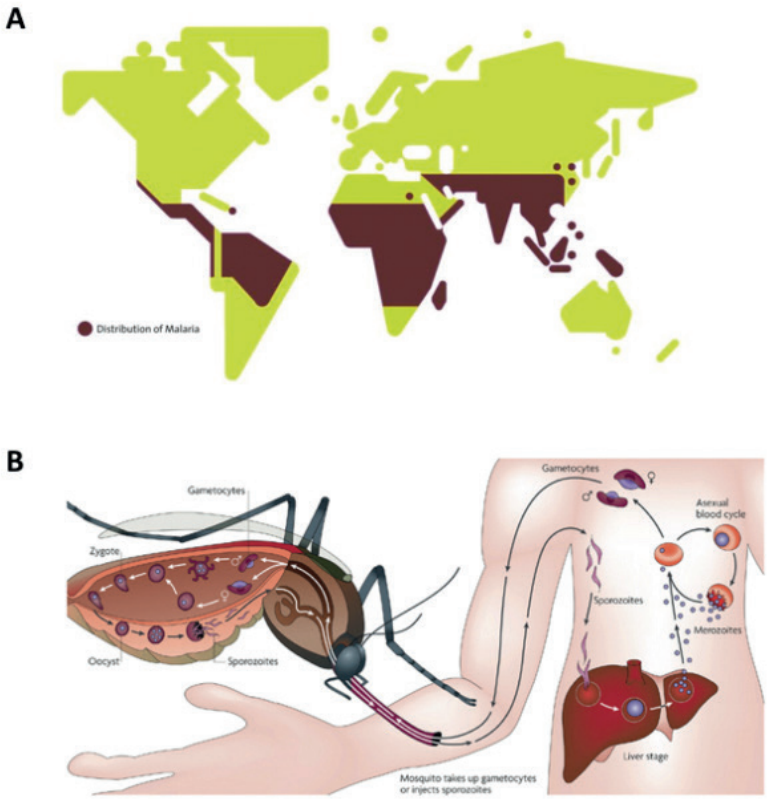

Figure 1. *P. falciparum* characteristics.
A Geographical dissemination of *P. falciparum* malaria, indicating specifically high incidence in sub-Sahara Africa. **B** Overview of the parasite life cycle, including the vector stage, liver stage, asexual and sexual blood stages.

MULTIDRUG RESISTANCE IN MALARIA

During the last few decades, many efforts have been made to create a safe and effective vaccine with the objective of malaria eradication; however, the complicated parasite life cycle and unravelled immune response mechanisms have been hampering these attempts, and clinical trials of the most promising vaccine have not resulted in an efficacy above 65%[6,7]. The development of a vaccine will most likely take many more years, and accessibility for people at risk as well as the efficacy of the product will remain a major challenge. In the meantime, conventional treatment of malaria infections has become increasingly problematic as resistance against long-term first-line antimalarials such as chloroquine, sulfadoxine-pyrimethamine, and mefloquine is broadly spread. Only a limited number of effective antimalarials are still available, which has led the World Health Organization to recommend

artemisinin-based combination therapies to ensure effective treatment and prevent further resistance acquisition. Nonetheless, a long-feared reduced *in vivo* susceptibility for artemisinin was reported in the Thai-Cambodian border and Western Cambodia in 2009[8], and although the K13-propellor domain was identified as a molecular marker[9], additional mutations contributing to resistance acquisition remain to be identified. In the case of quinoline resistance, active extrusion from the digestive vacuole by a mutated chloroquine resistance transporter (*Pf*CRT) gene inhibits accumulation at its target site[10]. Yet, mutations in *Pfcrt* alone cannot account for the variability in response to chloroquine treatment of different parasite clones[11]. Most likely other mechanisms contribute to resistance acquisition by decreasing drug pressure and creating a window for selection of resistant parasites, possibly by increased export of antimalarial compounds. Indeed, the ATP-binding cassette (ABC) transporter family member, multidrug resistance protein 1 (*Pfmdr1*), also named P-glycoprotein homologue (Pgh), has been associated with chloroquine and artemsinin resistance *in vitro*[12.] In the last two decades, many studies have concluded that mutations in *Pfmdr1* and an increased copy number of the *Pfmdr1* gene are indeed related to resistance against several types of drugs[12,13]. Likewise, single nucleotide polymorphisms in an ABC family member, multidrug resistance-associated protein 1 (*Pfmrp1*), have been linked with reduced drug response in malaria[11]. The role of many other ABC transport proteins has not yet been subject of investigation.

The ABC transport family

Many ABC transporters actively pump out a wide range of structurally and functionally diverse drugs, thereby decreasing intracellular drug accumulation, ultimately resulting in drug resistance[14]. The *P. falciparum* ABC family consists of 16 ABC members[15-17] that have been categorized into subfamilies (A through I) according to phylogenetic analysis of the primary or secondary structures of the conserved NBDs[17-19]. The structure of a typical ABC transporter is composed of two trans-membrane domains (TMDs), each consisting of 6 trans-membrane helices (TM), and two cytosolic nucleotide-binding domains (NBDs). ABC transporters are either encoded as full transporters (TMD-NBD-TMD-NBD) or as half transporters (TMD-NBD) that upon translation combine to form a functional unit. The TMDs contain one or more substrate-binding sites. ABC transporters, present in the plasma membrane in the inward-facing configuration, allow substrates to enter the putative substrate-binding chamber from the membrane or the cytoplasm **(Figure 2A)**[20]. Translocation of substrate across the membrane is achieved by rearrangement of the TM helices, physically closing them to the substrate delivery side and opening them on the opposite side of the membrane **(Figure 2B)**. This mechanism is induced by the binding of ATP and the subsequent dimerization

of the NBDs. ATP hydrolysis drives the NBDs apart, flipping the TMDs from the outward-facing to the inward-facing conformation to reset the cycle[20]. Our knowledge of the drug-binding sites is still rudimentary. For example, we lack detailed knowledge of the architecture of the drug-binding site, nor do we know how many sites an ABC transporter possesses, or how the hydrolysis of ATP is coupled to the binding and translocation of drugs across the membrane[14].

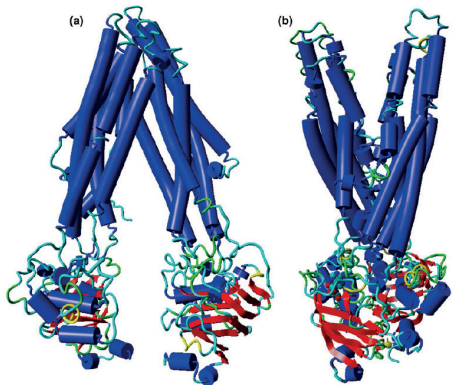

Figure 2. Crystal structures of ABC full transporters.
The transporter is composed of TMDs, each consisting of 6 TMs, and two cytosolic NBDs. **A** Front view of the nucleotide-free mouse multidrug exporter *Mdr1*A (3G5U.pdb) in the inward-facing conformation. **B** Side view of the nucleotide-bound Sav1866 multidrug exporter from *Staphylococcus aureus* (2HYD.pdb) in the outward-facing conformation. The structural figures were prepared using the program Yasara (www.yasara.org).

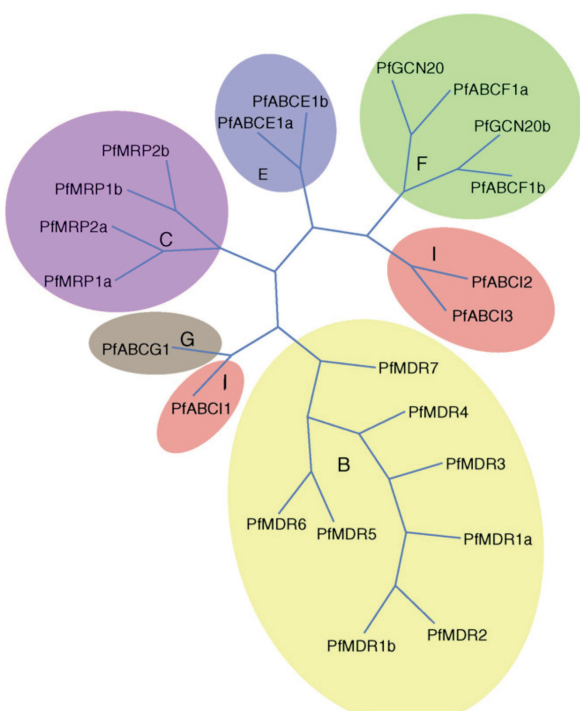

Figure 3. Phylogram of the *P. falciparum* ABC superfamily based on the NBD alignment.
The NBDs (30 amino acids before Walker A until Walker B) of the *Plasmodium falciparum* ABC proteins **(Table 1)** were aligned with ClustalW2[21] and the phylogenetic data were imported in TreeView (Page, 1996). In combination with blast searches the ABC transport proteins were categorized in subfamilies, as has been described previously[17].

In the *Plasmodium* parasite the ABC A subfamily is missing **(Figure 3)**, as is the case in all Apicomplexa. Seven members are present in the B subfamily, of which only *Pf*MDR1 is a full transporter. The C subfamily encodes two full transporters, whereas the G subfamily only contains one half transporter. Besides these proteins, there is one additional member in the ABC I subfamily that also encodes a TMD. Five ABC proteins do not contain TMDs and are members of the E, F, and I subfamily.

Pfmdr1 in multidrug resistance

*Pf*MDR1 is localized predominantly to the parasite's digestive vacuole membrane and to a lesser extent to the plasma membrane[22]. Indeed, it was demonstrated that *Pf*MDR1 transports the fluorescent compound Fluo-4 into the digestive vacuole[23].

In 1989 the *Pfmdr1* gene was shown to be amplified in some chloroquine-resistant parasites, indicating that it might be associated with *P. falciparum* chloroquine resistance[24]. However, when one of the two *Pfmdr1* copies of a drug-resistant strain were genetically disrupted, no change in chloroquine sensitivity was shown, but an increased in vitro susceptibility to mefloquine, lumefantrine, halofantrine, quinin and artemisinin was observed[25]. Analysis of *Pfmdr1* expression showed that the expression level is consistent with the gene copy number, but not associated with chloroquine sensitivity[25,26]. A genetic cross between chloroquine-resistant and -susceptible clones underlined that *Pfmdr1* amplification is not related to chloroquine resistance[27]. In fact, de-amplification of the *Pfmdr1* gene occurred after chloroquine pressure selection[28]. Meta-analysis of published data showed that an increased *Pfmdr1* copy number was correlated with mefloquine treatment failure[29]. Furthermore, three or more copies of *Pfmdr1* were also associated with recrudescence in patients treated with artesunate-mefloquine[30]. Indeed, in a culture adapted *P. falciparum* isolate, containing the *Pfmdr1* gene, copy numbers increased after stepwise increasing concentrations of mefloquine[31]. It was demonstrated that this event occurred frequently and can be expected to occur in most clinical cases. Parasites with multiple copies of the *Pfmdr1* gene, however, have decreased survival fitness in the absence of drug pressure[31].

Several *Pfmdr1* point mutations (N86Y, Y184F, S1034C, N1042D, and D1246Y) were demonstrated to be linked to decreased drug sensitivity[32]. The relationship between the *Pfmdr1* N86Y polymorphism and drug resistance has been investigated in more than 40 publications[29]. Systematic review and meta-analysis of published data showed that the *Pfmdr1* polymorphism

was associated with chloroquine and amodiaquine therapeutic failure[29]. In isolates from Thailand, artemether- lumefantrine pressure selects for the N86Y polymorphism[33]. In Tanzania, amodiaquine monotherapy can select for the N86Y/F184Y/D1246Y haplotype, whereas artemether- lumefantrine selects for the opposite haplotype[34]. Moreover, allelic replacement of the *Pfmdr1* gene provided proof that mutations S1034C, N1042D, and D1246Y can contribute to increased *in vitro* sensitivity for mefloquine, halofantrine, and artemisinin, whereas they confer resistance to quinine[12,13]. In addition, removal of these mutations can result in increased sensitivity towards chloroquine in a strain-specific manner[35]. In the absence of selective pressure, these mutations in the *Pfmdr1* gene also gave rise to a substantial fitness cost in the intraerythrocytic asexual stage of the parasite[36]. These polymorphisms are localized in different positions within the *Pf*MDR1 protein. N86Y is located in the first loop between TM1 and TM2; Y184F at the edge of TM3; S1034C and N1042D in TM11, whereas D1246Y is located in the C-terminal end of the protein.

Only very few multiple membrane spanning *Plasmodium* proteins have been expressed heterologously. Most likely the expression is rather difficult due to the A/T richness of the genes and the specific codon usage. *P. falciparum* genes that have been expressed successfully, usually encode for small soluble proteins or peptides[37]. Today, *Pfmdr1* is the only ABC transporter that has been functionally expressed. In 1994, *Pfmdr1* was first expressed in mammalian cells, and immunofluorescence revealed an intracellular localization of the transporter[38]. The authors demonstrated that wild-type *Pfmdr1* could mediate an increased intracellular accumulation of chloroquine and that this function is impaired in chloroquine-resistant mutants (S1034C and N1042D) of the protein. A follow-up study showed that *Pf*MDR1 does not transport chloroquine, but instead influences chloroquine accumulation by modulating the pH of acidic organelles, and it was speculated that the *Pfmdr1* gene encodes a vacuolar chloride channel[39]. Soon thereafter it was demonstrated that *Pfmdr1* expressed in yeast functions as a transport protein and complements a mutation in *ste6*, a gene encoding an ABC transporter that exports the mating pheromone a-factor[40]. Furthermore, *Pfmdr1* containing S1034C and N1042D appeared to abolish this mating phenotype, indicating that these polymorphisms reverse its transport function.

More than a decade later, chloroquine-sensitive *Pf*MDR1 ATPase activity was determined after heterologous expression of codon-optimized *Pfmdr1* in *Pichia pastoris*[41]. The single S1034C mutation was found to ablate drug stimulation of *Pf*MDR1 ATPase activity by quinine, mefloquine,

and chloroquine[42]. Recently, the functional expression was reported of *Pfmdr1* variants in Xenopus laevis oocytes[43]. This approach has led to the first direct evidence of antimalarial drug transport by *Pf*MDR1. Chloroquine, quinine and vinblastine, but not halofantrine, were found to be substrates of the wild-type (N86/Y184/S1034/N1042/D1246) *Pf*MDR1 transporter. By contrast, chloroquine and quinine were not transported by polymorphic *Pfmdr1* variants, whereas halofantrine and vinblastine were. Altered substrate specificity for mutated *Pfmdr1* transporters was thus demonstrated. Heterologous expression of *Plasmodium* transport proteins is an important tool that can identify substrates and effects of mutations on drug transport, which can be translated to drug resistance phenotypes. However, experiments in heterologous expression systems should be interpreted with caution when extrapolating conclusions from the model system towards the organisms under focus.

DRUG BINDING OF *Pf*MDR1

The x-ray structure of mouse MDR1 in a drug-binding-competent state was described recently. This protein has 27% amino acid identity with *Pf*MDR1 and therefore can be used to generate a homology model in which the polymorphisms can be visualized and related to specific protein functions. The multiple substrate binding sites that were visualized in the crystal structure show that it is difficult to predict at which position in the pocket drugs will bind. Combining the homology model with drug-interaction, mutagenesis, and docking studies can indicate the precise location of drug binding. This strategy can also be applied to other *Pf*ABC family members involved in multidrug resistance. Development of new antimalarials might benefit from these new developments.

Combining the various transport and drug sensitivity data directs towards a more or less plausible mechanism of multidrug resistance by *Pfmdr1*. Wild-type *Pfmdr1* expressed in *Xenopus laevis* oocytes readily effluxes chloroquine and quinine, whereas polymorphisms can abolish this transport[43]. The opposite is true for halofantrine; wild-type *Pf*MDR1 does not transport the drug, whereas introduction of specific polymorphisms creates a halofantrine-transporting *Pf*MDR1. This is in agreement with the observation that wild-type *Pfmdr1* stimulates intracellular accumulation of chloroquine and that this process is inhibited by the introduction of specific single nucleotide polymorphisms[38]. These polymorphisms also affect the interaction of chloroquine with the

ATPase activity of *Pf*MDR1[42]. Furthermore, the main localization of *Pf*MDR1 in the membrane of the digestive vacuole indicates that it could transport antimalarials from the cytosol of the parasite into this vacuole, where the drug target of the quinoline antimalarials is assumed to be situated. As expected from these characteristics, the introduction of the polymorphisms results in a chloroquine- and quinine-resistant, but halofantrine-sensitive, parasite[12,13,35]. Moreover, in chloroquine-resistant strains, wild-type *Pfmdr1* might be down-regulated[28], and up-regulation of wild type *Pf*MDR*1* does not lead to resistance against chloroquine, whereas mefloquine resistance was observed[29]. These facts imply that the transporter has a crucial role in uptake of chloroquine and possibly quinine into the digestive vacuole, which may herald a shift in the wide-spread assumption that quinolines adequately enter the digestive vacuole through simple diffusion[44]. Apart from the parasite strain, substrate specificity, drug dosing, rate of efflux, drug interactions and competition are complex processes that could explain some paradoxical results reported in literature. Also the translation from ATPase activity to the actual translocation of a substrate complicates clear conclusions towards substrate identification. Moreover, the physiological role of *Pf*MDR1 is not known, and drugs might inhibit *Pf*MDR1 transport activity, thereby reducing uptake of other substrates required in the digestive vacuole for survival of the parasite.

OTHER *Pf*MDR TRANSPORTERS

Of the 11 *P. falciparum* ABC family members that contain a TMD, the ABC B subfamily is the largest with seven members **(Table 1)**. Only *Pf*MDR1 is a full transporter, whereas the other six are half transporters. *Pf*MDR2 is localized in both the plasma membrane[45] and the digestive vacuole membrane[46] of the parasite. Initially, *Pf*MDR2 was assumed to be involved in chloroquine resistance[47], but in a later study that showed equal protein levels of *Pf*MDR*2* in chloroquine-resistant and -sensitive parasites, this suggestion was dismissed[45]. *Pf*MDR2 is probably not involved in drug sensitivity, but seems to be an efflux pump of heavy metals. Rosenberg *et al*. developed a *P. falciparum* line resistant against the heavy metals cadmium and lead[48]. When exposed to these metals no accumulation was observed in this line, whereas a sensitive line did accumulate the metals. It was shown that this sensitive line harboured a truncated inactive *Pf*MDR*2* protein, and the resistant line a full-length active protein. Also in a study employing fluorescently labelled cadmium, cadmium accumulation was significantly decreased in the resistant line[49]. These findings strongly suggest that the *Pf*MDR2 protein acts as an efflux pump of cadmium and lead. Furthermore, the F423Y polymorphism in *Pfmdr2* has been associated with *in vitro* pyrimethamine

resistance[50]. For *Pfmdr5*, only the plasma membrane expression in all asexual stages of *P. falciparum* has been reported[17].

A trinucleotide insertion in *Pfmdr6* showed a significant association with decreased sensitivity to chloroquine[11], which was later also confirmed for artesunate[51]. Recently, length variations in simple repeats of *Pfmdr6* have been associated to altered sensitivity towards dihydroartemisinin *in vitro*[52]. In another study on polymorphic repeats, an association with piperaquine and lumefantrine sensitivity was found[53].

To our knowledge, no results have been published on the characteristics of *Pfmdr3*, *Pfmdr4*, or *Pfmdr7*. It would be worthwhile to investigate the *Pfmdr*s in more detail, as these transporters are candidate drug transporters.

*Pf*MRPS IN MULTIDRUG RESISTANCE

The *P. falciparum* parasite encodes for two MRP transport proteins, which are both full transporters. A few years ago, single nucleotide polymorphisms in *Pfmrp1* were linked to decreased sensitivity to chloroquine and quinine in *P. falciparum*[11]. Also *Pfmrp2* was associated with drug sensitivity more recently **(Table 1)**.

Whereas *Pf*MDR1 is localized mainly in the membrane of the digestive vacuole[22], plasma membrane expression of *Pf*MRP1 and *Pf*MRP2 in all asexual stages of the parasite was demonstrated[17,54]. Expression of *Pfmrp1* and *Pfmrp2* was up-regulated by mefloquine and chloroquine in laboratory cultures of both drug-sensitive and -resistant strains[55], and alterations in expression profiles for both *Pfmrp1* and *Pfmrp2* were observed upon exposure to different dosages of mefloquine[56].

Table 1. The *Plasmodium* ABC superfamily

Family	*Name*	*P.falciparum*	*P. vivax*	*P. knowlesi*	*P. berghei*	*P. yoelli*	*P. chabaudi*
ABCB							
ABCB1	*Pfmdr1*	PF3D7_0523000	PVX_080100	PKH_100920	PBANKA_123780	PY00245	PCAS_123820
ABCB2	*Pfmdr2*	PF3D7_1447900	PVX_118100	PKH_125840	PBANKA_131170	PY06054	PCAS_131500
ABCB3	*Pfmdr3*	PF3D7_1145500	PVX_092880	PKH_094310	PBANKA_090350	PY06546	PCAS_070470
ABCB4	*Pfmdr4*	PF3D7_0302600	PVX_119255	PKH_083760	PBANKA_040120	PY03961	PCAS_040210
ABCB5	*Pfmdr5*	PF3D7_1339900	PVX_082915	PKH_121420	PBANKA_135330	PY02551	PCAS_135790
ABCB6	*Pfmdr6*	PF3D7_1352100	PVX_083495	PKH_120190	PBANKA_136480	PY07089	PCAS_136940
ABCB7	*Pfmdr7*	PF3D7_1209900	PVX_084520	PKH_130880	PBANKA_060830	PY01826	PCAS_060890
ABCC							
ABCC1	*Pfmrp1*	PF3D7_0112200	PVX_097025			PY05035	PCAS_144600
ABCC2	*Pfmrp2*	PF3D7_1229100	PVX_124085	PKH_144590	PBANKA_144380		
ABCE							
ABCE1		PF3D7_1368200	PVX_115370	PKH_110230	PBANKA_114410	PY04219	PCAS_114360
ABCF							
ABCF1		PF3D7_0813700	PVX_123085	PKH_142470	PBANKA_142380		PCAS_142560
ABCF2		PF3D7_1121700	PVX_091705	PKH_091910	PBANKA_092660		PCAS_091770
ABCG							
ABCG1		PF3D7_1426500	PVX_085205	PKH_132390	PBANKA_101810	PY00207	PCAS_101790
ABCI							
ABCI1		PF3D7_0319700	PVX_095250	PKH_082130		PY00170	PCAS_121950
ABCI2		PF3D7_1434000	PVX_084835	PKH_131530	PBANKA_101080	PY06911	PCAS_101070
ABCX							
ABCX1		PF3D7_1413500	PVX_085775	PKH_133410	PBANKA_102920		PCAS_102900

A number of polymorphisms of *Pfmrp1* were related with altered antimalarial sensitivity; the K1466R and the I876V polymorphisms in *Pfmrp1* were selected among parasites in recrudescent infections after sulfadoxine-pyrimethamine and artemether-lumefantrine treatment, respectively[57,58]. Furthermore, the F1390I SNP was associated with artemisinin, mefloquine and lumefantrine sensitivity in vitro[59], the I876V polymorphism was related to reduced chloroquine sensitivity in samples isolated at the China-Myanmar border[60] and 191Y and 1390I SNPs were associated with increased sensitivity towards chloroquine and quinine[61]. Furthermore, *Pfmrp1* polymorphisms in parasite isolates from North-East Myanmar showed reduced in vitro susceptibility towards chloroquine, mefloquine, pyronaridine and lumefantrine[62], and decreased sensitivity towards mefloquine and artesunate was found for a number of polymorphisms in this gene[63]. Combined, these data indicate that *Pf*MRP1 is involved in *P. falciparum* drug sensitivity.

Pfmrp1 disruption in a *P. falciparum* chloroquine-resistant line showed that under normal culture conditions these parasites could not grow to a parasitemia higher than 5%, possibly because of lower efficiency in removing toxic metabolites[64]. The disrupted parasite also accumulated more radiolabeled glutathione, chloroquine, and quinine. Moreover, they became more sensitive to multiple antimalarial drugs, including chloroquine, quinine, artemisinin, piperaquine, and primaquine. This suggests that *Pf*MRP1 plays a role in the efflux of glutathione, chloroquine, and quinine.

For the *Pfmrp2* gene, repeat polymorphisms were associated to altered lumefantrine sensitivity[53]. A deletion in the upstream region of the gene which induces *Pf*MRP2 expression was associated with increased resistance against quinoline antimalarials[65]. A study of Veiga *et al*. showed a high diversity in *Pfmrp2* polymorphisms, of which a number was related to decreased sensitivity of again quinoline antimalarials[66].

OTHER *PLASMODIUM* ABC TRANSPORTERS IN MULTIDRUG RESISTANCE

The location of the *Plasmodium* ABC transport proteins within the parasite are depicted in **Figure 4**. In addition to the seven ABC B and two ABC C subfamily members, there are seven other ABC members **(Table 1)**. Two of these, ABCG1 (PF3D7_1426500) and ABCI1 (PF3D7_0319700), contain a TMD and thus most likely are transport proteins.

The role of the *Plasmodium* ABCG1 in multidrug resistance has not yet been investigated. The human G family member, breast cancer resistance protein (BCRP/ABCG2), can actively extrude a broad range of endogenous and exogenous substrates (e.g. cytostatics) across biological membranes[67]. The orthology of the *Plasmodium* ABCG1 transport protein and the human BCRP makes it an interesting candidate drug transporter. *Pfabcg1* (indicated as *Pfabcg2* in[67]) was disrupted in a quest for novel transmission-blocking antimalarial compounds[68]. The resulting parasite developed normally during asexual replication, however, gametocyte formation was strongly reduced.

ABCI3 is an atypical ABC family member that does not possess a clear ABC signature motif, but according to a Pfam search, ABCI3 belongs to the ABC family[69]. In conclusion, next to the *P. falciparum* B and C subfamily members there is another good candidate drug transporter in the G subfamily, and in the I subfamily a possible transport protein with little homology to mammalian transporters.

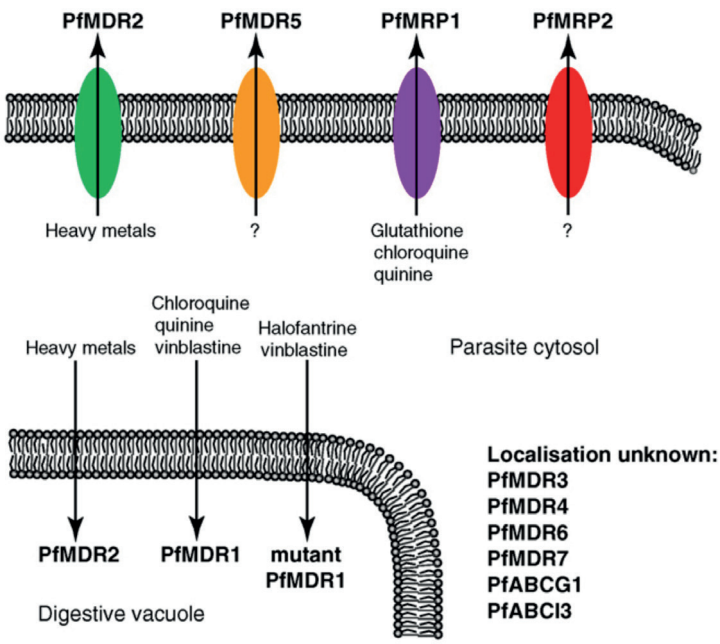

Figure 4. Schematic representation of the localisation of the *P. falciparum* ABC transporters in the parasite.
The *Pf*ABC transport proteins are indicated on the parasite plasma membrane and digestive vacuole membrane. In addition the known substrates are depicted.

ABC TRANSPORTERS IN OTHER *PLASMODIUM* SPECIES

Of the four human malaria parasites, *P. falciparum* is the most virulent species. Outside Africa *P. vivax* is very frequent and represents a major threat to health[70]. *P. ovale* and *P. malariae* generally give mild infections[71]. There is little information about ABC transporters in *P. vivax* and to our knowledge no information about these proteins in *P. ovale*, *P. malariae* and *P. knowlesi*. In **Table 1**, the ABC family members of the human malaria parasite *P. vivax*, the primate malaria parasite *P. knowlesi* and the rodent malaria parasites *P. berghei*, *P. yoelii*, *P. chabaudi* are compared with *P. falciparum*. In general the same family members are present, however, in the MRP family, rodent species encode only one MRP orthologue.

The *mdr1* gene was also identified in the human malaria parasite *P. vivax*, but initially no correlation was found between chloroquine resistance and specific mutations[72]. A few years later, the Y976F mutation of *Pvmdr1* was associated with reduced susceptibility to chloroquine[73], but greater susceptibility to arsenate and mefloquine[74]. However, in Madagascar this mutation was already fixed in the *P. vivax* population and no significant correlation between the *Pvmdr1* Y976F mutation and chloroquine treatment outcome was observed[75]. Furthermore, the supposedly selective pressure on this mutation was investigated in multiple malaria endemic settings, however, no direct effect on chloroquine sensitivity could be identified[76-81]. Also for the F1076L mutation a resistance mechanism was not observed[77,82,83]. Interestingly, the single nucleotide polymorphisms found in *P. vivax* differed from those present in *P. falciparum*, indicating that differences other than mutations may be implicated in chloroquine resistance in these two malaria parasite species[84,85]. Increased *Pvmdr1* gene copy number has been detected[79,81], which was not associated with chloroquine resistance[73,77,] but was associated with a reduced susceptibility to mefloquine[74,80]. *Pvmdr1* gene amplification appeared significantly more common in an area where mefloquine pressure has been intense compared to areas where there has been less exposure of parasites to mefloquine[84.] Furthermore, increased transcript levels were found in a patient with severe *P. vivax* malaria as compared to patients with mild malaria[86]. These first studies on *Pv*MDR1 indicate that this transporter is involved in drug transport and susceptibility of the parasite. More research on *Pv*MDR1 and the other *Pv*ABC transport proteins is required to value the importance of these transporters in *P. vivax* multidrug resistance, and to determine their physiological functions.

AIM AND OUTLINE OF THIS THESIS

In this thesis, we will investigate the function of ABC transporter proteins in *Plasmodium* species, to elucidate their contribution to decreased drug sensitivity and their physiological role in cellular processes. Increased knowledge of the specific mechanism of action of ABC transporters in antimalarial drug sensitivity may potentially aid in the rescue of known compounds and the design of new drugs. Insights into (essential) biological functions of these proteins could reveal novel drug targets, or contribute to the development of alternative antimalarial strategies. In **Chapter 2** we will focus on the interaction of antimalarial compounds with human ABC transport proteins by using a vesicular inhibition assays. These experiments indicate that some antimalarial compounds inhibit ABC transport proteins at therapeutic concentrations, which can potentially lead to harmful drug-drug interactions. In **Chapter 3** we will aim at the identification of substrates of *Pf*MRPs to elucidate their physiological function and assess the contribution of transport of specific substrates to drug sensitivity of the parasite. This is done through an untargeted metabolomics approach and LC/MS-MS validation in parasites in which the *mrp*-encoding genes are stably deleted. These parasites are subsequently assessed in **Chapter 4** for their antimalarial sensitivity. In order to evaluate essential functions of MRP transporter proteins, the effect of *mrp* deletion is studied in all stages of the parasite life cycle in both *P. berghei* and *P. falciparum*. Finally, we focus on MDR transport proteins in **Chapter 5**, where we target all encoded transporters for gene deletion in *P. berghei* and evaluated parasite viability throughout the life cycle, and also assess targetable transporters in *P. falciparum*. In **Chapter 6**, we evaluate the implications of our findings and including a future perspective on ABC transport protein research in *Plasmodium* parasites. Targeting the interaction of the parasite with its host environment through ABC transport proteins, either by extrusion of toxic waste products or protection against host metabolites, might be an important new weapon in the battle against malaria.

CHAPTER 2

Atovaquone and quinine antimalarials inhibit ATP Binding Cassette transporter activity

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ABSTRACT

Background Therapeutic blood plasma concentrations of anti-malarial drugs are essential for successful treatment. Pharmacokinetics of pharmaceutical compounds are dependent of adsorption, distribution, metabolism, and excretion. ATP binding cassette (ABC) transport proteins are particularly involved in drug deposition, as they are located at membranes of many uptake and excretory organs and at protective barriers, where they export endogenous and xenobiotic compounds, including pharmaceuticals. In this study, a panel of well-established anti-malarial drugs which may affect drug plasma concentrations was tested for interactions with human ABC transport proteins.

Methods The interaction of chloroquine, quinine, artemisinin, mefloquine, lumefantrine, atovaquone, dihydroartemisinin and proguanil, with transport activity of P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), bile salt export pump (BSEP) and multidrug resistance-associated proteins (MRP) 1–4 were analysed. The effect of the anti-malarials on the ATP-dependent uptake of radio-labelled substrates was measured in membrane vesicles isolated from HEK293 cells overexpressing the ABC transport proteins.

Results A strong and previously undescribed inhibition of BCRP-mediated transport by atovaquone with a 50% inhibitory concentration (IC_{50}) of 0.23 μM (95% CI 0.17-0.29 μM) and inhibition of P-gp-mediated transport by quinine with an IC_{50} of 6.8 μM (95% CI 5.9-7.8 μM) was observed. Furthermore, chloroquine and mefloquine were found to significantly inhibit P-gp-mediated transport. BCRP transport activity was significantly inhibited by all anti-malarials tested, whereas BSEP-mediated transport was not inhibited by any of the compounds. Both MRP1- and MRP3-mediated transport were significantly inhibited by mefloquine.

Conclusions Atovaquone and quinine significantly inhibit BCRP- and P-gp-mediated transport at concentrations within the clinically relevant prophylactic and therapeutic range. Co-administration of these established anti-malarials with drugs that are BCRP or P-gp substrates may potentially lead to drug-drug interactions.

INTRODUCTION

ATP binding cassette (ABC) transporters are membrane-bound proteins that allocate a wide variety of compounds at the expense of ATP, even against steep concentration gradients[87]. P-glycoprotein

(P-gp/ABCB1), bile salt export pump (BSEP/ABCB11), multidrug resistance-associated proteins (MRP1-4/ABCC1-4), and breast cancer resistance protein (BCRP/ABCG2) are among the most important drug transporters of the ABC protein family. ABC transport proteins are known for their capacity to protect the organism from potentially toxic xenobiotics through excretion, thereby decreasing intracellular concentrations. Indeed, typical localization of these export transporters are at the blood-brain barrier, placenta, gut, and at the apical side of liver and kidney cells. Two compounds may interact with the same transport protein through induction of expression, inhibition of protein function or competition of substrates. Pharmacokinetics of co-administered drugs can be critically altered when drug-drug interactions occur at the level of the ABC transport proteins, as distribution and selective excretion of these compounds may depend heavily on ABC protein-mediated transport. This can be reflected either in unexpected high blood plasma concentrations potentially causing toxic effects, or subtherapeutic concentrations at the site of action, diminishing therapeutic effects.

It is essential to assure effective blood plasma concentrations upon treatment with anti-malarial compounds in order to cure severely ill patients and prevent resistance acquisition through exposure of the parasite to sublethal blood plasma concentrations. The first-line treatment as recommended by the World Health Organization (WHO) currently consists of artemisinin-based combination therapy[88]. However, resistance against these regimens has been detected and the number of anti-malarials that can be subsequently applied are limited[8]. Toxic effects by unintended elevated blood plasma concentrations, however, should also be avoided.

Direct interaction with ABC transporter capacity of anti-malarial compounds has not been explored in detail. In vitro assays have indicated a possible effect on P-gp mediated transport or expression after exposure to chloroquine, quinine, mefloquine, primaquine, amodiaquine, piperaquine, artemisinin and dihydroartemisinin, however, contradictory conclusions concerning the interaction of anti-malarial compounds with ABC transport proteins could be drawn from different experimental set-ups[89-94]. A possible interaction of antimalarial compounds with MRP-type transporters and BCRP has also been described[95-98]. Co-administration of anti-malarial compounds with other drug-types is highly anticipated. For instance, human immunodeficiency virus (HIV) and malaria co-infections are likely to occur, as there is a high overlap in geographical dissemination[99]. Therefore, the effect of antimalarial compounds on ABC-mediated transport capacity should be explored in more detail in order to secure the most effective treatment strategies for patients receiving multiple drug regimens.

In this study the direct interaction of a panel of eight well-known anti-malarial compounds (chloroquine, quinine, artemisinin, mefloquine, lumefantrine, atovaquone, dihydroartemisinin and proguanil) with transport activity of P-gp, MRP1-4, BCRP and BSEP in a vesicular overexpression transport assay have been analysed. Anti-malarials (100 μM) that caused a decrease in substrate transport larger than 66.7% were further characterized to determine their 50% inhibitory concentrations (IC_{50}). Potent and previously undescribed inhibition of BCRP-mediated transport by atovaquone and P-gp-mediated transport by quinine was observed at concentrations within their therapeutic range.

METHODS

Materials

[6,7-^{3}H(N)]Estrone-sulphate ammonium salt ([^{3}H]-E1S, specific activity 45.6 Ci/mmol), Tauro[carbonyl-3H]Cholic Acid sodium salt ([^{3}H]TCA) (5 Ci/mmol) and [6,7-^{3}H(N)]Estradiol 17-β-D-glucuronide ([^{3}H]-$E_2$17βG) (34.3 Ci/mmol) were purchased from PerkinElmer Life and Analytical Sciences (Groningen, Netherlands). [^{3}H(N)]-methyl quinidine ([^{3}H]-NMQ) (80 Ci/mmol) and unlabelled NMQ [N-methyl-quinidine] were purchased from Solvo Biotechnology (Szeged, Hungary). Bac-to-Bac and Gateway systems, Dulbecco's modified Eagle's medium, GlutaMAX-I culture medium, and foetal calf serum were purchased from Life Technologies (Bleiswijk, Netherlands). Primers were purchased from Biolegio (Nijmegen, Netherlands), and a plasmid purification midiprep kit was from Genomed (Löhn, Germany). Triple flasks (500 cm^2) were purchased from Sanbio BV Biological Products (Uden, Netherlands). Estradiol 17-β-D-glucuronide ($E_2$17βG), estrone-sulphate (E1S), taurocholic acid (TCA) adenosine 5'-triphosphate magnesium salt (bacterial source), goat-anti-mouse IgG antibody IRDye 800 and goat-anti-rabbit Alexa 680 secondary antibodies, chloroquine (CQ), quinine (Q), artemisinin (ART), mefloquine (MQ), lumefantrine (L), atovaquone (ATO), dihydroartemisinin (DHA) and proguanil (PG) were purchased from Sigma-Aldrich (Zwijndrecht, Netherlands). Protein concentrations were determined with a Bio-Rad protein assay kit from Bio-Rad Laboratories (Veenendaal, Netherlands), and 96-well filter plates were purchased from Millipore (Etten-leur, Netherlands).

Baculovirus generation

Human P-gp, BCRP, BSEP and MRP1-4 had previously been cloned into the Gateway pDONR221 vector. Sequences matched accession numbers NM_000927, NM_004827, NM_003742,

NM_004996, NM_000392, NM_00378, and NM_005845 respectively[100-104]. Some sequences did hold silent mutations of described polymorphisms. Gateway cloning was used to transfer the genes into a VSV-G improved pFastBacDual vector for mammalian cell transduction. The production of baculovirus was executed according to the Invitrogen Bac-to-Bac manual.

Cell culture and transduction

HEK293 cells were grown to 40% confluency in Dulbecco's modified Eagle's medium-GlutaMAX-I containing 10% foetal calf serum at 5% CO_2 in 500 cm^2 triple flasks. Culture medium was removed and 25 mL of medium combined with 10 mL virus was added and incubated at RT for 20 min, followed by the addition of another 40 mL of complete medium including 5 mM sodium butyrate to enhance protein expression.

Membrane vesicle isolation and protein analysis

Cells were harvested three days post transduction by a 5 min centrifugation step at 3,000 *g*. Cells were resuspended in ice-cold hypotonic buffer (0.5 mM sodium phosphate, 0.1 mM EDTA, pH 7.0) containing protease inhibitors (100 mM phenylmethylsulfonyl fluoride, 5 mg/ml aprotinin, 5 mg/ml leupeptin, 1 mg/ml pepstatin and 1 mg/ml E-64) and shaken at 4°C for 30 min. This lysate was centrifuged 100,000 *xg* for 30 min at 4°C, after which the pellet was homogenized in ice-cold TS buffer (10 mM Tris-HEPES and 250 mM sucrose, pH 7.4) supplemented with protease inhibitors described before using a tight-fitting Dounce homogenizer for 25 strokes. Two subsequent centrifugation steps at 4°C of firstly 20 min at 4000 *g* followed by supernatant centrifugation for 60 min at 100,000 *g* ensured harvesting of the membrane fraction. The pellet was resuspended in ice-cold protease free TS buffer and passed 25 times through a 27-gauge needle to enhance membrane vesicle formation. Protein concentration in these vesicles was determined using the Bio-Rad protein assay, vesicles were flash-frozen in N_2 and stored at -80 °C.

Vesicular transport assays

A rapid filtration technique that has been described earlier was applied to evaluate uptake of transporter specific substrates into the vesicles; NMQ for P-gp, E1S for BCRP, $E_2$17βG for MRP1-4 and TCA for BSEP[105]. Briefly, 0.015-0.15 µCi of labelled substrate was combined with unlabelled substrates to a concentration of 0.1-1 µM in a 30 µL reaction mixture with 4 mM ATP, 10 mM $MgCl_2$ and 7.5 µg total protein membrane vesicles in TS buffer. Transport was allowed by transfer of the

plates to 37 °C during 1-5 min, a time-point within the linear phase of time-dependent transport, as previously determined[100-104]. Hereafter, the reaction was rapidly stopped by placing the plates back on ice and the addition of 150 µL ice-cold TS buffer. Samples were subsequently transferred to a 96-well filter plate that had been pre-incubated with TS buffer, and filtered using a multiscreen HTS-vacuum manifold filtration device (Millipore). Filters were washed and extracted, after which 2 mL scintillation fluid was added to each filter. Radioactive signal on the filters was determined by liquid scintillation counting. Negative controls included eYFP-transduced vesicles and AMP instead of ATP in the reaction mixture.

In the first screen, all anti-malarial compounds were added to the reaction mixture to evaluate transport inhibition at a concentration of 100 µM. Solvents were used as negative controls, as CQ was dissolved in milliQ, Q and ART in methanol, MQ, L, ATO and DHA in DMSO and PG in 50% ethanol. When ATP-dependent uptake was reduced more than 66.7%, the compound was considered a potential inhibitor, and multiple concentrations were tested in the reaction mixture to determine the IC50 value. All concentrations were tested in duplicates or triplicates in two individual biological replicates containing vesicles of independent transductions. Results were depicted and statistically analysed using Graphpad Prism, version 5.03. IC_{50} values were determined by nonlinear regression analysis of (log) inhibitor-response curves with variable slope. Maximal transport was restricted to 100%, and the minimum was set to be equal or greater than 0%. Statistical analysis was performed using IBM SPSS Statistics 20, applying one-way ANOVA (Analyis of variance).

RESULTS

Inhibitory profile of anti-malarials against ABC transporter activity

The inhibitory characteristics against the ABC transporters of eight well-known antimalarials; CQ, Q, ART, MQ, L, ATO, DHA and PG, was investigated at at a 100 µM concentration. For each transporter protein, specific radio-labelled substrates were applied to measure ATP-dependent transport into the vesicular overexpression system; N-methyl quinidine (7nM radio-labelled diluted with 90 nM non-radio-labelled) for P-gp, estrone sulphate (74 nM) for BCRP, estradiol 17-β-D glucuronide (150 nM) for MRP1-4 and taurocholic acid (1 µM) for BSEP[101-104].

A significant inhibitory effect of 100 μM CQ, Q, MQ and PG on P-gp-mediated NMQ transport was observed. CQ reduced NMQ transport to 50% ($p < 0.001$) and PG to 76% ($p < 0.001$), whereas Q and MQ gave more pronounced inhibitory effects to 15% ($p < 0.001$) and 30% ($p < 0.001$) P-gp-mediated NMQ transport, respectively. ART and DHA slightly induced transport activity to 131% ($p < 0.001$) and 112% ($p = 0.033$), respectively **(Figure 1A)**. All anti-malarials inhibited BCRP-mediated estrone sulphate transport activity at 100 μM concentrations. Most potent inhibitors were MQ, ATO and PG, which reduced estrone sulphate transport to 8.5%, 22% and 36% with $p < 0.001$, respectively **(Figure 1B).** CQ reduced transport to 69%, Q to 45%, ART to 62%, L to 44%, and DHA to 70% of solvent-exposed BCRP-mediated transport capacity ($p < 0.001$). Significant inhibition of taurocholic acid transport by BSEP was observed for ATO, which reduced uptake to 54% ($p < 0.001$) and MQ, which reduced uptake to 72% ($p = 0.037$). Furthermore, induction of BSEP transport activity was found for CQ (117%, $p < 0.001$), ART (117%, $p < 0.001$) and DHA (114%, $p < 0.001$) **(Figure 1C)**. MQ was found to have a modest but significant inhibitory effect on estradiol 17-β-D glucuronide transport by MRP1 as this was reduced to 50% ($p < 0.001$), whereas ATO was observed to induce this process to 141% ($p < 0.001$) **(Figure 1D).** Induction was also observed for ART and ATO on MRP2-mediated estradiol 17-β-D glucuronide transport to 151% ($p = 0.015$) and 162% ($p = 0.020$), respectively. However, no significant inhibition was measured for any of the anti-malarials tested (Figure 1E). MRP3-mediated translocation of estradiol 17-β-D glucuronide was significantly inhibited by MQ at a 100 μM concentration to 70% ($p = 0.001$), whereas ART and DHA induced this process to 122% ($p = 0.016$) and 121% ($p = 0.020$), respectively (Figure 1F). No significant estradiol 17-β-D glucuronide transport inhibition of MRP4 could be detected (**Figure 1F**). As the 100 μM concentration is not within the physiological range of compound exposure, the most potent inhibitors were selected for further investigation. Inhibition of Q and MQ on P-gp-mediated transport, as well as BCRP inhibition by MQ, ATO and PG, were studied in more detail to determine their potencies.

Determination of inhibitory potency of strong inhibitors

Subsequently, transport inhibition assays were performed for a larger concentration range of Q, MQ, ATO and PGP to evaluate P-gp or BCRP activity. Inhibition of transport was measured in a similar fashion applying the same specific radio-labelled substrates. Drug concentrations were logarithmically depicted, and a sigmoidal, inhibitor-response, variable slope equation was fitted to the data to determine the inhibition curve. Maximal inhibition to 0% transport was not always reached, which might be due to endogenous transport present in the vesicular membranes.

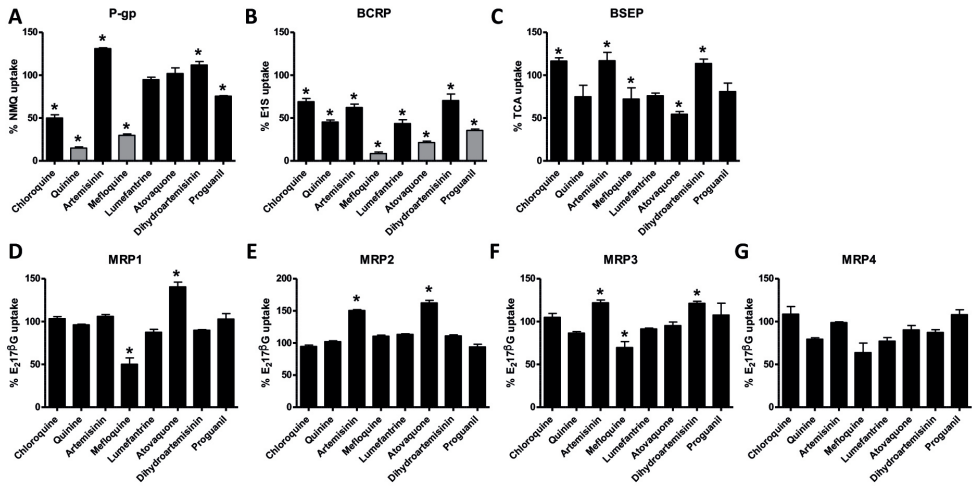

Figure 1. Inhibitory effect of anti-malarial drugs on ABC transport activity.
The inhibitory effect of 100 μM of CQ, Q, ART, MQ, L, ATO, DHA and PG on ABC transporter activity was assessed. Transport was measured in pmol/mg protein/min and expressed as percentage of solvent controls, which represent 100% transport. Bars with * are significantly different from solvent controls, $p < 0.05$. **A** P-gp-mediated transport of NMQ was significantly inhibited by CQ, Q, MQ and PG, and increased by ART and DHA. **B** BCRP-mediated transport of E1S was significantly inhibited by all compounds, most pronounced inhibitors were MQ and ATO. **C** BSEP-mediated transport of TCA was significantly inhibited by ATO, but not by the other anti-malarials. Induction of transport was observed for CQ, ART and DHA. **D-G** MRP1-4-mediated E217βG transport. MQ significantly inhibited MRP1 and MRP3 transport activity. Furthermore, induction of MRP1 mediated transport was found for ATO, which, together with ART, also stimulated MRP2 transport activity. MRP3 mediated transport was stimulated by both ART and DHA. Inhibition larger than 66.7% was found for Q and MQ on P-gp transport, as well as MQ, ATO and PG on BCRP transport activity (highlighted bars).

The strongest inhibitory effect for ATO on BCRP-mediated transport was found at median nanomolar range. Transport of estrone sulphate was inhibited with 50% by this compound at 0.23 μM (95% CI 0.17-0.29 μM) **(Figure 2A)**, whereas MQ and PG required the addition of 18 μM (95% CI 17–20 μM) **(Figure 2B)** and 118 μM (95% CI 93–148 μM) **(Figure 2C)** to achieve a similar effect on BCRP activity, respectively. Also for the other compound-transporter combinations, IC_{50} values were found in the low to median micromolar range. The effect of Q on P-gp-mediated NMQ transport inhibition was the strongest, and the IC_{50} was defined at 6.8 μM (95% CI 5.9-7.8 μM) **(Figure 2D)**. MQ was a less potent inhibitor with an IC_{50} of 72 μM (95% CI 49–104 μM) **(Figure 2E)**. The inhibitory concentration of ATO and Q transport were within the therapeutic range of blood plasma concentrations after both prophylactic and curative anti-malarial dosing.

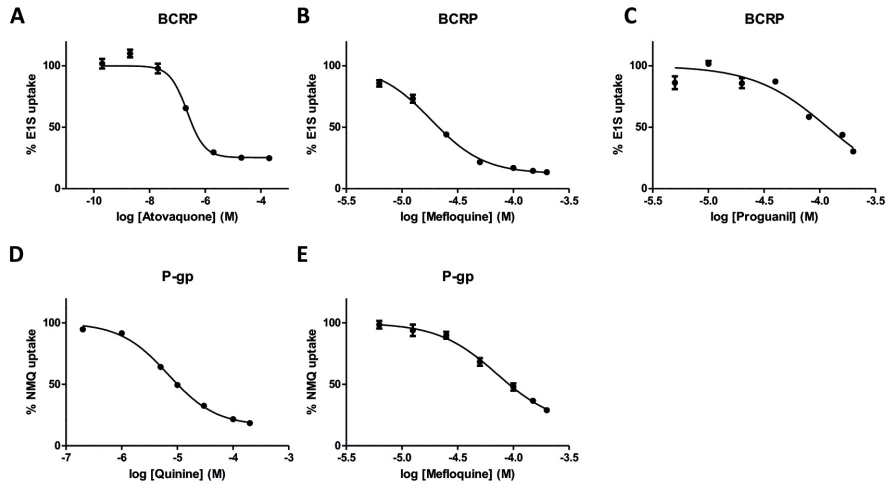

Figure 2. Concentration-dependent inhibition of potent anti-malarial inhibitors of P-gp and BCRP. The inhibition of NMQ transport by P-gp was determined for **A** MQ and **B** Q. BCRP activity was inhibited according to the dose–response curves for **C** MQ **D** ATO and E PG.

DISCUSSION

In this study, the interaction of anti-malarial compounds CQ, Q, ART, MQ, L, ATO, DHA and PG with the activity of P-gp, BCRP, MRP1-4 and BSEP ABC transport proteins were investigated. ATO was found to be a strong inhibitor of BCRP-mediated transport, which has not been described previously. Also Q was identified as a potent inhibitor of P-gp-mediated transport. In addition, subtle alterations on transporter activity have also been identified for other compound-transporter combinations, both inhibitory and stimulating. These interactions can be either competitive or non-competitive. Allosteric interactions that stimulate transport have been observed for several ABC transporters and are substrate dependent, due to which translation of these results to other transporter-substrate combinations is difficult.

A 50% inhibition of BCRP-mediated transport activity could be achieved with 0.23 µM ATO. This concentration is easily reached in blood plasma during both prophylactic and therapeutic use of ATO, as maximal ATO blood plasma concentrations are around 14 µM (range 8-26 µM) after a daily prophylactic dose of 250 mg[106]. Although the free concentration of ATO is reduced due to its high plasma protein binding, intracellular concentrations at the target site may be higher. ATO is used in a fixed combination with PG in Malarone®, which is prophylactively prescribed to travellers,

and at higher dosages to treat falciparum malaria especially in regions of ACT failure[88,107]. BCRP is located primarily on the apical side of excretory organs, and it is highly involved in excretion of xenobiotics from the body[108,109]. As ATO is excreted into bile against steep concentration gradients, involvement of ABC transport proteins such as BCRP is likely[110] and interactions with ATO can occur when elimination of co-administered therapeutics is inhibited.

Indeed, cases of interactions with ATO have been reported. The azithromycin AUC (area under the curve) and maximal concentrations were lower in all patients when taken in combination with ATO by HIV-1 positive children[111]. Although direct interaction of azithromycin with BCRP has not been investigated, interaction at this level cannot be excluded. Moreover, a clear increase in plasma concentration of etravirine, a reverse transcriptase inhibitor, and saquinavir, a protease inhibitor, was observed in a Caucasian female who started malaria prophylaxis with ATO/PG (250/100 mg) fixed dose combination[112]. Etravirine and saquinavir were prescribed to treat HIV1 subtype B in an antiretroviral combination therapy, supplemented with raltegravir and maraviroc. AUCs during a 12-hour measurement interval were increased 55% for etravirine and 274% for saquinavir, and peak concentrations after administration of the antiretrovirals was markedly increased. Saquinavir and etravirine have previously been described as potent BCRP inhibitors, but not substrates, with IC50 concentrations of 19.5 and 1.0 μM[113,114]. Both raltegravir and maraviroc do not inhibit BCRP, indicating that interaction with BCRP is specific for saquinavir and etravirine[115]. An alternative or complementary explanation could be interaction at the level of Cytochrome P450 (CYP) enzymes, as PG is mainly metabolized by CYP2C19 but also partly by CYP3A4, saquinavir by CYP3A4 and etravirine mainly by CYP3A4 and to a minor extent by CYP2C9 and CYP2C19[116-118]. Raltegravir is not metabolized by members of the CYP family, however, maraviroc is a substrate of CYP3A4[119,120]. A strong correlation at this level of drug interaction could therefore not be observed, stressing the plausible role of transporter-mediated drug interactions.

Another study demonstrated a significant decrease in ATO plasma concentration when taken in combination with efavirenz, lopinavir/ritonavir or atazanavir/ritonavir therapy[121]. Interaction at the level of metabolism through glucuronidation was proposed. However, as atovaquone is only marginally glucuronidated but mostly excreted unchanged into the bile, interaction at the level of ABC transport proteins and more specifically BCRP could play an important role[110]. Indeed, efavirenz, lopinavir and atazinavir have been described as inhibitors of BCRP-mediated transport[122]. Lopinavir

and efavirenz were found to be stronger inhibitors, and correspondingly, ATO concentration was decreased more drastically in these two combinations compared to atazinavir co-administration.

Other pharmaceuticals that interact with BCRP-mediated transport are fluoroquinolone antibiotics, kinase inhibitors, cytostatics, antifolates and statins[123-129]. Interactions with ATO therapy might be anticipated when co-administered. These drugs are not widely used in malaria-endemic areas, however, interactions with prophylactic doses of ATO used by travellers can be anticipated.

A 50% inhibition of P-gp-mediated transport by Q was found at a concentration of 6.8 μM. Indeed, in other in vitro cellular uptake experiments Q has been described to be both an inhibitor and a substrate of P-gp[89,93,94,130-133]. The concentration at which Q was effective was lower in the current study than previously described. Most likely this can be attributed to the difference in substrates used. Maximal plasma concentrations reach 30 μM during a seven day regimen of 10 mg/kg oral dose three times daily of quinine sulphate, and although Q is bound to plasma-proteins to some extent, clinically relevant interactions at the level of P-gp-mediated transport during quinine treatmen may be expected[134].

Interactions with Q have been described for ritonavir/lopinavir combination therapy as well as ritonavir monotherapy, and for nevirapine, rifampicin, cyclosporine and digoxin. Q co-administration with digoxin decreased billary excretion of the latter, indicating specific involvement of transport processes[135]. When co-administered with ritonavir, Q blood plasma concentrations were increased[136]. Ritonavir indeed is both a substrate and inhibitor of P-gp, therefore interaction at this level may explain the increase in Q concentration[137,138]. After rifampicin, nevirapine and lopinavir co-administration, Q blood plasma concentrations were decreased[134,139-141]. Rifampicin interacts with P-gp as substrate, inhibitor and inducer, and lopinavir has been found to inhibit P-gp[142-144]. However, this has not been shown for nevirapine. Q is one of the oldest anti-malarial drugs still in use, and although it is not used any more in first line treatment strategy, its use has increased as it is often applied as an alternative treatment after ACT stock-outs[145]. Furthermore, for treatment of malaria infections in pregnant women it is one of the few compounds that can be applied safely[146]. Adherence to this compound is known to be low due to the large range of common and often plasma concentration-dependent side effects[147]. For these reasons, establishing effective but non-toxic blood plasma concentrations is essential in the treatment of malaria, and interaction with co-administered compounds that mediate P-gp transport should be tightly monitored.

Especially, the interaction of both ATO and Q with antiretroviral medication could have severe implications on treatment strategies for both infections, as HIV is another major contributing factor to morbidity, especially in sub-Saharan regions of Africa[99]. Many different antiretroviral compounds are being prescribed, depending on personal characteristics and resistance status, and for many of these compounds interactions with BCRP have been described.

CONCLUSIONS

Anti-malarial compounds can reduce ABC transporter activity. ATO appeared to be a potent inhibitor of BCRP and Q of P-gp in vitro. Both compounds inhibited ABC transporter activity at concentrations equalling prophylactic and effective blood plasma concentrations. Potential involvement in interactions with antiretroviral and antibiotic compounds have been described for ATO and Q, which can be explained by the observed inhibitory effects on BCRP and P-gp transport activity.

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CHAPTER 3

MRP1 mediates folate transport and antifolate sensitivity in *Plasmodium falciparum*

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ABSTRACT

Multidrug Resistance-associated Proteins (MRP) of *Plasmodium falciparum* have been associated with altered drug sensitivity. Knowledge on MRP substrate specificity is indispensible for the characterization of resistance mechanisms and identifying its physiological roles. An untargeted metabolomics approach detected decreased folate concentrations in red blood cells infected with schizont stage parasites lacking expression of MRP1. Furthermore, a tenfold decrease in sensitivity towards the folate analogue methotrexate was detected for parasites lacking MRP1. *Pf*MRP1 is involved in the export of folate from parasites into red blood cells and is therefore a relevant factor for efficient malaria treatment through the folate pathway.

INTRODUCTION

ATP Binding Cassette (ABC) proteins are membrane bound transporters known for their capacity to translocate a wide variety of compounds at the expense of ATP[87,148]. ABC export proteins have important physiological roles in homeostasis and signaling processes, but also contribute to resistance acquisition by lowering intracellular concentrations of curative drugs. Indeed, in *Plasmodium falciparum* (*P. falciparum*), single nucleotide polymorphisms and copy number variations of especially *Pfmdr1* (PF3D7_0523000) and *Pfmrp1* (PF3D7_0112200) have been associated with anti-malarial resistance. The multidrug resistance-associated proteins (MRPs) are members of the ABC subfamily C, and are typically involved in transport of organic anions including glutathione-, glucuronide- or sulfate-conjugated compounds from endogenous and exogenous origin. MRP1 has been localized at the plasma membrane of *P. falciparum* parasites during intra-erythrocytic development[17]. Increased expression of this gene has been associated with mefloquine and chloroquine resistance, and single nucleotide polymorphisms in *Pfmrp1* with *in vivo* selection after sulfadoxine/pyrimethamine and artemether/lumefantrine treatment[55,57,58]. Furthermore, transient deletion of this gene in a chloroquine resistant parasite strain resulted in increased sensitivity towards chloroquine, quinine, artemisinin, piperaquine and primaquine.

Besides unraveling the contribution of ABC transporters to anti-malarial resistance, the identification of MRP substrates can shed light on essential functions in biological processes of the parasite. The resistance portfolio of *P. falciparum* against anti-malarials now includes first-line artemisinin derivatives,

and as alternative treatments are scarce, evidently new compounds targeting parasite-specific processes are urgently required[8,9,88]. ABC transport proteins are attractive drug targets, as they are often expressed on the easily accessible plasma membrane, play key roles in biological processes and are involved in transportation of drugs to or from their targets site[149].

Heterologous expression of ABC transport proteins has been proven successful at identifying substrates for many different organisms. *Pfmdr1* has been expressed in *Pichia pastoris* and in *Xenopus laevis* oocytes after codon optimization[41,43]. Point mutations in this gene altered substrate specificity, resulting in decreased transport of a number of anti-malarial compounds. Despite the use of codon-optimized or codon-harmonized sequences, correct and functional orthologous expression of *Pf*MRP1 could not be achieved by us after multiple attempts. Therefore, we have been unable to determine the substrate specificity of *Pf*MRP1 in direct transport assays.

Applying metabolomics to tissues and body fluids of wild type and knockout mice has proven to be a powerful approach to identify new substrates of (orphan) ABC transporters[150]. Compounds that are differentially distributed are identified using a metabolite database. In this manner, composition of urine, plasma and bile derived from wild type mice could be compared with that of knockout mice and specific ABC substrates were found.

In this study, we aimed to identify new endogenous MRP1 substrates by applying untargeted metabolomics on erythrocytes infected with wild type and *Δmrp1 P. falciparum* parasites.

MATERIALS AND METHODS

Parasite culture, gene deletion and selection of iRBC

Stable deletion of *mrp1* was exerted via the double crossover recombination method previously described[151]. From these gene removal procedures, two gene-deleted lines from independent transfections were obtained: *PfΔmrp1* and *PfΔmrp1+hdhfr*. In the *PfΔmrp1* lines, the positive selectable marker that was used during the deletion procedure was removed through a FLPe recombinase procedure, which has not been performed in the *PfΔmrp1+hdhfr* gene deleted line[152]. This selectable marker consists of the human dihydrofolate reductase (*hdhfr*) gene, which confers resistance to and enables selection with the antifolate compound WR99210 for parasites

in which the gene targeting construct has been integrated. Parasites were cultured in a semi-automated culture system using standard in vitro culture conditions for *P. falciparum* as previously described[9,16,153]. In short, human serum supplemented RPMI medium was changed twice daily and 0,5% hematocrit shaken cultures were maintained at parasitaemias below 20%. Schizont stage parasites were selected through synchronization of the parasite cultures using a 63% percoll centrifugation at 2.000xg, and approximately 40 hrs after initial synchronization, a second percoll treatment was performed to select late stage schizonts only[154]. The RBCs with the selected parasite stages were washed with medium and counted in a Burker-Türk counting chamber, after which they were centrifuged, washed with PBS, and finally lysed in hypotonic buffer (5mM KH^2PO^4, pH 7,4). Parasites and RBC membranes were removed with a second centrifugation step at 4.000xg for 5 minutes, after which the supernatant was used for further analysis.

Identification of MRP substrates by untargeted metabolomics

Untargeted metabolomics was performed on RBC lysate as described previously[155]. In short, 250 µL of the RBC lysate was selected, and 750 µL methanol was added for deproteinization. Samples were vortexed, centrifuged and the supernatant evaporated in a speed-vac. Extracts were reconstituted in 50 µL 50% methanol, and HPLC was performed on a Dionex Ulti-Mate 3000 RSLCnano system (Thermo Fisher Scientific) using a ReprosilPur Basic C18 HPCL column protected by a KrudKatcher Ultra in-line filter (Phenomenex). Mass spectrometry was performed using an LTQ-Orbitrap Discovery mass spectrometer (Thermo Fisher Scientific), operated in negative ionization mode. Data acquisition was performed using Xcalibur software (Thermo Fisher Scientific). Data were analyzed using XCMS Online (xcmsonline.scripps.edu)[156,157] after conversion of chromatograms to mzXML format using Proteowizard[156,158]. XCMSonline settings for feature detection: Polarity, negative; method, centWave; ppm, 2.5; snthr, 20; peakwidth, 15-90; mzdiff, 0.01; prefilter peaks, 3; prefilter intensity, 100; noise, 0; On average we detected 2203 features per sample (range: 1738-2801). XCMS online retention time correction settings: method, obiwarp; profStep, 0.1. XCMS online alignment settings: method, density; bw, 5; mzwid, 0.015; minfrac, 0.5; minsamp, 1. Accurate masses of differentially present compounds were used to search in the online databases HMDB and METLIN and their identity confirmed by making MS-MS fragmentation spectra. Relative metabolite concentrations were determined from ion chromatograms through manual peak integration after correction for parasite density[159,160]. Each parasite line was tested in triplicate, and two measurements were performed for every sample.

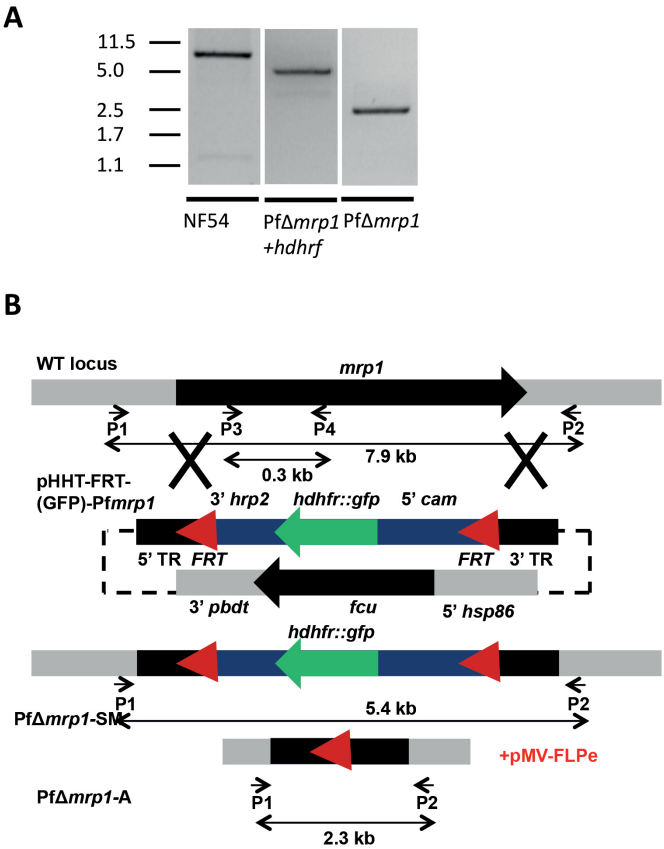

Figure 1. *Pfmrp1* knockout confirmation
A Long range PCR was performed using primers P1 and P2, spanning the 5′ and 3′ TR, amplifying the targeted domain of mixed blood stage parasites. A PCR product for wild type mrp1 of 7937 bp was obtained, band sizes of 5353 and 2257bp were obtained for the *PfΔmrp1+hdhfr* and *PfΔmrp1* lines, respectively. Stable deletion of the **B** *mrp1* genes in *P. falciparum* was accomplished through double crossover recombination. Target regions (TR) at the 5′ and 3′ end of the gene allowed insertion of the disruptive vector and excision of the ORF upon positive selection of transfected parasites containing the hdhfr selection cassette, followed by negative selection of single crossover events using the fcu marker. Upon consecutive transfection with the FLPe encoding construct, selectable marker sequences between the FRT sites were excised, resulting in a gene deleted parasite with only a 34 base pair FRT site scar.

Detection of folate by LC/MS-MS

Folate concentrations in the red blood cell department of parasitized cells were measured using a LC-MS/MS system consisting of a HPLC (Accela®, Thermo Scientific, San Jose, CA, USA), a quaternary ultra high pressure pump, a vacuum degasser and an autosampler, coupled to a (TSQ Vantage®, Thermo Scientific, San Jose, CA, USA) triple quadrupole mass spectrometer. Liquid Chromatographic (LC) separation was performed using a HSS T3 analytical column

(1.8 µm; 100 × 2.1 mm, Acquity UPLC®, Waters, Ireland) coupled to a VanGuard® HSS T3 pre-column (1.8 µm; 5 × 2.1 mm, Acquity UPLC®, Waters, Ireland). The elution gradient used consisted of 30 seconds 100% A, 3 minutes 15% B, followed by 7 minutes 100% A. Solvent A of the mobile phase contained 0.1% (v/v) formic acid (HCOOH) in water and solvent B 0.1% (v/v) formic acid (HCOOH) in acetonitrile. The column temperature was set at 30°C, sample injection volume was 10 µL and total analysis run time 10 minutes. The effluent from the HPLC was passed directly into the electrospray ion source, where capillary temperature was set at 207°C and vaporizer temperature at 382°C. Heated electrospray ionization (HESI) in the positive mode was achieved at a spray voltage of +3.5kV, with nitrogen as sheath and auxiliary gas with a gas pressure of 20 and 15 AU (Arbitrary Units) respectively. Argon was used as collision gas at a pressure of 1.5 mTorr. During the LC–HESI–MS/MS analysis, a time-segment program was developed to switch the divert valve of the mobile phase to waste or detection mode to prevent ion suppression and contamination of the ion source. The positive ion mode was used with selected reaction monitoring (SRM) for the quantitative analysis of folic acid. The most abundant product ion was used for quantification, which was performed using peak areas. A second and third product ion were used for qualification purposes. The optimal SRM transitions and collision energies (CE) were determined according to **Table 1**.

Table 1. SRM Transition and Collision Energies

Compound	Precursor Ion (m/z)	Product Ion (m/z)	CE (eV)
Folic acid	442.1	295.0	17
Folic acid qualifier 1	442.1	119.9	35
Folic acid qualifier 2	442.1	176.0	37

Antifolate sensitivity assays

Blood stage parasites were routinely cultured non-synchronously. Drug sensitivity assays were performed in 100 µL sample formats in black low binding, clear-bottom 96-well culture plates. Cultures were started at 1% parasitaemia, 2,5% haematocrite in RPMI medium supplemented with 10% human serum. Antifolate compounds that were tested were pyrimethamine, proguanil, methotrexate, WR99210 and trimethoprim. Pyrimethamine, methotrexate and WR99210 were purchased from Sigma; proguanil and trimethoprim were obtained from the Medicines for Malaria Venture organization (www.mmv.org/malariabox). Samples were incubated for 72 hours in candle jars, after which samples were frozen in order to lyse cells and parasites. Each compound

concentration was tested in triplicate, with three replicates for compounds that differentially affected the gene deleted lines, and two for compounds that did not shift IC_{50} values for mutant parasites. Parasite growth and viability was measured through determining parasitic lactate dehydrogenase (pLDH) concentration, as described previously[154,161]. To each well, 70 µL reaction mixture containing 286 mM sodium lactate, 286 µM APAD, 357,5 µM resazurin and 5,66 U/mL diaphorase was added, starting a reaction in which pLDH hydrolyses APAD+ into APADH, at the expense of lactate which is converted into pyruvate. APADH on its turn is reduced to obtain the fluorescent compound resorufin after resazurin transformation. The reaction was incubated on room temperature for 60 minutes, after which resorufin concentration was measured on a Synergy 2 (Bio-Tek, USA) fluorescent plate reader at 530 nm excitation and 590 nm emission settings. Solvent or lowest drug concentrations exposed parasite samples were set at 100% growth, and samples with maximal inhibition concentrations were equalized to 0% growth. The inhibition curve fitting and statistical analysis of IC_{50} concentrations were performed in GraphPad Prism, version 5.03. Curves were fitted using an inhibitor response (variable slope) calculation, and slopes were adjusted to NF54 steepness. Maximal inhibition was fitted to greater than 0%.

RESULTS

The *PfΔmrp1* and *PfΔmrp1+hdhfr* lines were evaluated on correct gene deletion. Diagnostic long range PCR spanning the integration site was performed and *mrp1* amplification resulted in the expected band sizes of 5353 bp in the *PfΔmrp1+hdhfr* mutant line, and 2257 bp for the *PfΔmrp1* mutant line in which the hdhfr selectable marker was removed **(Figure 1A)**. PCR amplification of this gene of NF54 wild type genomic DNA resulted in a band size of 7937 bp. A schematic overview of the production of these mutant lines is depicted in **Figure 1B.**

Untargeted metabolomics was applied on parasites lacking MRP1 expression, to identify new endogenous substrates. We found that a compound with an m/z ratio of 440.1326 and a retention time of 19 minutes was less abundant in lysates of erythrocytes infected with schizonts lacking MRP1 expression than in erythrocytes infected with wild type schizonts **(Figure 2A-C)**. Database searches putatively identified this compound as folate, which was subsequently confirmed by comparing its fragmentation spectra with that of folate present in DMEM culture medium. No additional differentially distributed compounds could be detected using this approach, and also

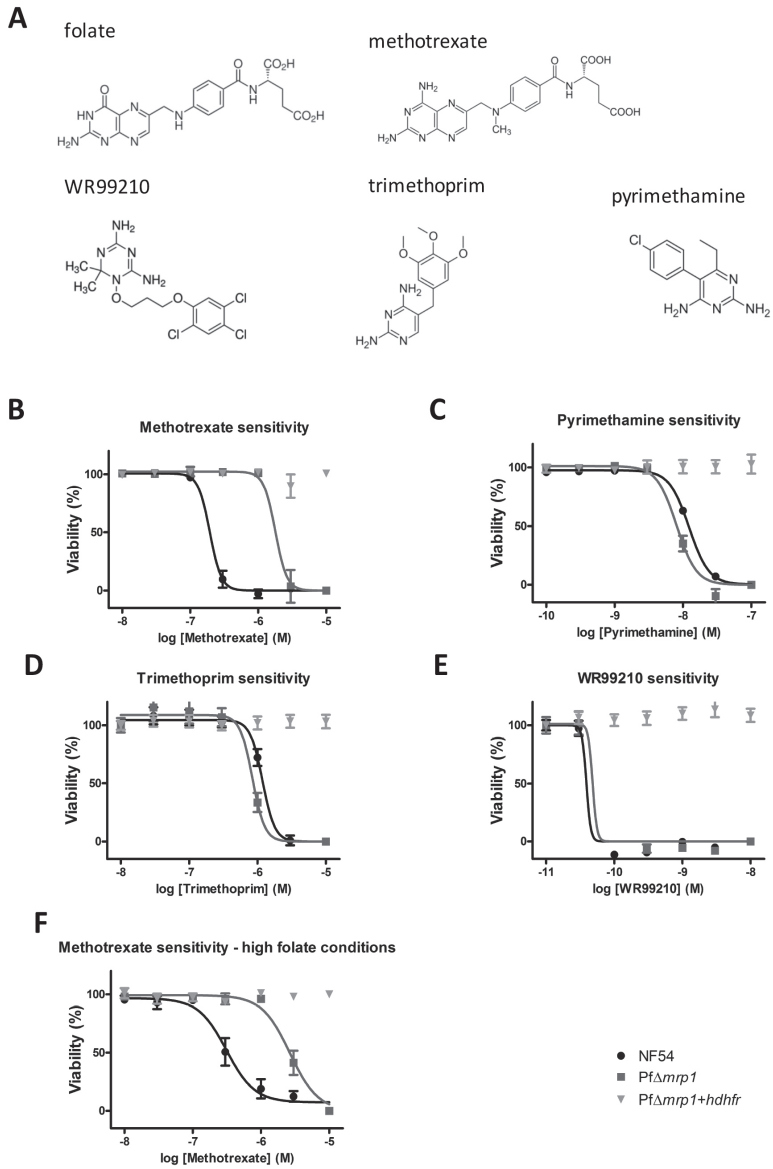

Figure 3. Sensitivity of *Pfmrp1* deleted parasites towards anti-folate compounds
A Molecular structures of antifolate compounds that were used in drug sensitivity assays; **B** methotrexate, **C** pyrimethamine, **D** trimethoprim and **E** WR99210. For methotrexate, a tenfold decrease in sensitivity for the *PfΔmrp1* parasites could be observed. No significant decrease in IC_{50} could be detected for *PfΔmrp1* gene deleted parasites compared to NF54 wild types for the other anti-folate compounds that were tested. A small increase in sensitivity was observed for pyrimethamine in the *PfΔmrp1* parasites. **F** Methotrexate sensitivity was tested in culture medium with a tenfold increase in folic acid concentration, to 23 µM. Sensitivity of NF54 wild type parasites towards this antifolate was significantly decreased.

folate concentrations were not decreased in erythrocytes infected with ring and trophozoite stage parasites lacking MRP1 expression.

For confirmation the of the results obtained with the untargeted metabolomics approach, we used a LC/MS-MS method to specifically detect folate in the erythrocyte lysates. Also with this targeted approach a significant decrease in folate concentrations of *PfΔmrp1* infected red blood cells was detected **(Figure 2D)**. Mean folate concentrations in NF54 infected red blood cells were detected at 3.4 µM (95% CI 2.1-4.8), whereas measurements in *PfΔmrp1* infected cells resulted in a mean concentration of 1.2 µM (95% CI 0.5-2.1), which is lies within the reference range for uninfected red blood cells (0.32-1.4 uM).

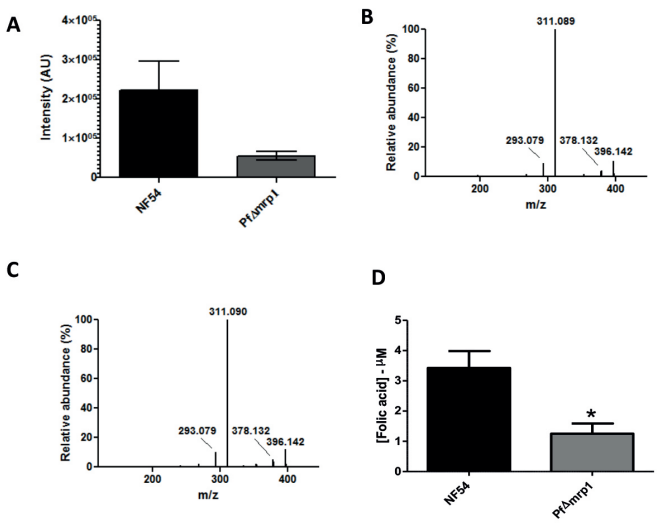

Figure 2. Decreased folate concentrations in red blood cells infected with *Δmrp1* schizonts
A Area under the curve measurement revealed a decreased abundance in red blood cells infected with *Δmrp1* schizonts for a compound with a /z ratio of 440.1326 and a retention time of 19 minutes **B** The fragmentation spectrum of the compound showed clear similarity of that of folate (**C**) **D** Folic acid concentrations were measured using LC/MS-MS in the red blood cell compartment of cells infected with NF54 wild type parasites or the *PfΔmrp1* strain. Erythrocytes infected with NF54 wild type parasites were found to contain 3.43 µM (95% CI 2.11-4.75) folic acid, whereas measurements in *PfΔmrp1* infected cells resulted in a significantly decreased concentration of 1.25 µM (95% CI 0,453-2.05).

We hypothesized that MRP1 acts as a direct or indirect folate efflux mediator, and cytosolic folate concentrations of the parasite are most likely influenced by the deletion of the gene encoding *mrp1*. Cytosolic folate concentrations can potentially alter the effect of anti-folates, which are commonly used in anti-malarial treatment. Therefore, we tested the sensitivity of the *mrp1* deleted lines towards a panel of well-known antifolates; methotrexate, pyrimethamine, trimethoprim,

and WR99210 **(Figure 3A)**. *PfΔmrp1* parasite sensitivity towards methotrexate was decreased significantly with a tenfold shift **(Figure 3B, Table 2)**. For the other anti-folate compounds that were tested, no significant decrease in IC_{50} was detected for *PfΔmrp1* parasites compared to NF54 wild type **(Figure 3C-F, Table 2)**. Only for pyrimethamine, a small but significant increase in sensitivity was observed. In all cases the sensitivity of the *PfΔmrp1+hdhfr* line towards this class of compounds was measured as control. Consequently to retaining the *hdhfr* resistance marker, these parasites were resistant towards treatment with anti-folates.

Folate concentrations measured in the red blood cell compartment were slightly higher (3.4 μM) than in the culture medium (2.3 μM). In order to assess the contribution of an increased folate concentration to the decrease in anti-folate sensitivity, the methotrexate drug sensitivity assays were repeated with a tenfold higher folic acid concentration in culture medium (23 μM). Methotrexate sensitivity significantly decreased for NF54 wild type parasites **(Figure 3F, Table 2).** For the *PfΔmrp1* line a smaller and insignificant decrease in sensitivity was observed.

Compound	NF54 (95% CI)	PfΔmrp1 (95% CI)
Methotrexate	190 (160-230)	1800 (1200-2500)
Methotrexate - high folate	310 (230-420)	2700 (2100-3400)
Pyrimethamine	12 (11-13)	8.1 (7.0-9.4)
Trimethoprim	1200 (1100-1300)	840 (680-1000)
WR99210	0.039 (0.029-0.053)	0.048 (0.000-1800)

Table 2
Drug sensitivity assays (IC_{50} in nM).

DISCUSSION

Mutation and upregulation of ABC transporters have been associated with *P. falciparum* drug resistance. However, direct identification of substrates has been hampered by difficulties in cloning and heterologous expression[37,162]. In this study, we have applied untargeted metabolomics to identify MRP1 substrates using wild type and gene-deleted parasites.

The metabolite profile of infected red blood cells was analyzed, and significantly decreased concentrations of folate were detected in infected cells when the *Plasmodium mrp1* was deleted. These results were further confirmed by a targeted approach, that also showed a decrease in

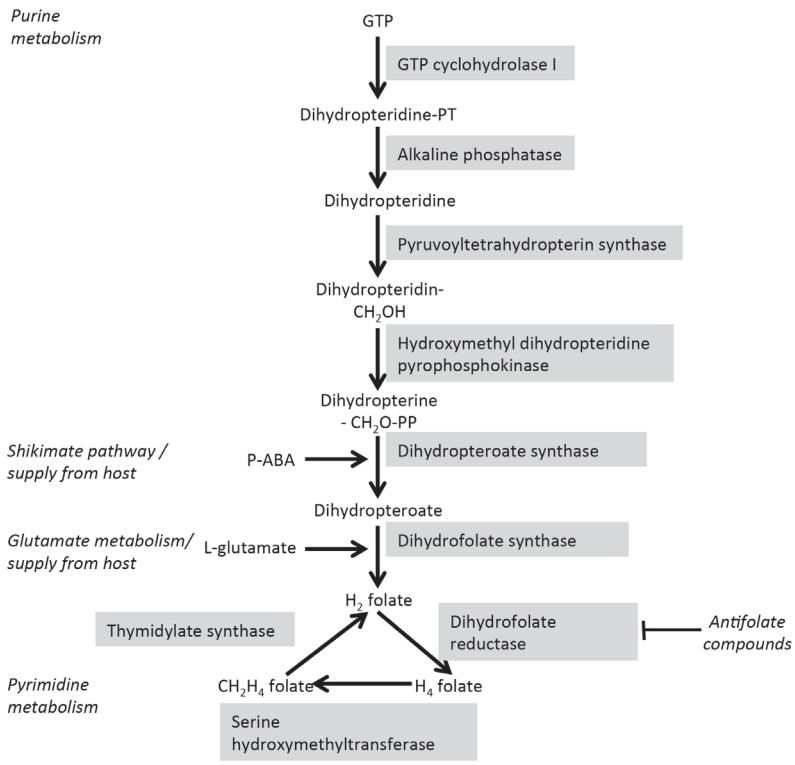

Figure 4. Folate biosynthesis pathway in *Plasmodium* parasites
Plasmodium parasites can synthesize folate *de novo* through the Shikimate pathway or through salvage of host pABA or CH3-THF. Folate antagonists act either during *de novo* synthesis, where the pABA and hydroxymethyl dihydropteridine pyrophosphate are used to form dihydrofolate or during conversion of dihydrofolate to tetrahydrofolate (DHFR) enzyme. Adapted from [189,190].

folate concentration in *PfΔmrp1* infected red blood cells. This indicates that MRP1 is involved in folate export, and we hypothesized that anti-folate sensitivity might be altered for *mrp1*-deleted parasites, as intraparasitic folate concentrations might be affected. Indeed, decreased sensitivity of the *PfΔmrp1* parasites towards methotrexate was detected, however, the effectivity of other anti-folate compounds including pyrimethamine, WR99210 and trimethoprim was not different in *mrp1*-gene deleted parasites compared to NF54 wild type parasites. Although *PfΔmrp1* parasites were initially selected by applying the antifolate WR99210, the selection marker (human *dhfr*) had been removed and parasite culture had been prolonged without the addition of selective drugs prior to these experiments. In addition, another ABC transporter knockout parasite that was selected using the same method did not show an indication of decreased folate levels (data not shown).

Folate is a known substrate of MRP transport proteins in many organisms, including humans[163-166.] Also in *P. falciparum*, MRP1 has previously been suggested to be a folate exporter, which was based on the identification of specific point mutations in the encoding gene after prolonged antifolate (sulfadoxine/ pyrimethamine) pressure in the population[57]. *Plasmodium* parasites can synthesize folate *de novo* through the Shikimate pathway or through salvage of host para-aminobenzic acid (pABA) or 5-methyltetrahydrofolate (CH3-THF) imported from extracellular compartments **(Figure 4)**[167,168]. The conservation of *de novo* synthesis enzymes underlines the high demand for folate during specific life cycle stages in which proliferation of the parasite is unparalleled[169]. Although folate is involved in multiple processes, its function as one-carbon donor in purine synthesis is most fundamental and explains its high consumption during cellular replication[170,171]. Folate antagonists act upon schizont stage parasites in which DNA replication peaks, and inhibit folate synthesis at two levels; either during *de novo* synthesis, where the pABA and hydroxymethyl dihydropteridine pyrophosphate components are joined into dihydrofolate by the hydroxymethyl dihydropteridine pyrophosphokinase – dihydropteroate synthase enzyme complex, or during conversion of dihydrofolate to tetrahydrofolate by the dihydrofolate reductase (DHFR) enzyme. Sulfadoxine, sulfalene, sulfamethoxazole and dapsone are structural analogues of pABA. The sulfa-adducts that are incorporated with these compounds lead to enzyme activity inhibition in downstream folate metabolism. DHFR is the target of a second set of anti-malarial antifolates; pyrimethamine, proguanil, methotrexate, trimethoprim and WR99210. These compounds in general bind to DHFR with high affinity, thereby inhibiting its function. Methotrexate is the only structural analogue of folate.

We have evaluated the efficacy of multiple antifolate compounds on *mrp1*-deleted *P. falciparum* parasites. The IC_{50} concentrations that were obtained in this study are within the range of previously detected in vitro sensitivity profiles. For pyrimethamine, the IC_{50} value of 12 nM lies within the range of reported inhibitory concentrations (<10 to 480 nM) in different assay types[172-177.] Also for WR99210, the IC_{50} of 0.039 nM was very near to those previously reported for different parasite lines (0.075-0.21 nM)[178-180]. Trimethoprim IC_{50} concentrations that have been reported to range from 130 nM to 30 µM in different assay types of chloroquine sensitive and resistant strains in medium depleted of folic acid and pABA are in harmony with our observation (1,2 µM)[180-182]. For methotrexate, sensitivity reports range between 0.32 nM and 600 nM in in vitro assays, which is in agreement with our NF54 IC_{50} measurement of 190 nM[183-185.]

Only for methotrexate we found a MRP1 specific effect on parasite sensitivity as the IC_{50} was increased by tenfold after deletion of *mrp1*. As folate concentrations are presumably increased in *mrp1*-deleted parasites resulting from reduced folate export, the affinity of methotrexate for DHFR may be antagonized by the increased intraparasitic folate concentrations. The intraparasitic folate concentrations could, however, not be determined due to the very small sample volume. The beneficial effect of increased folate concentrations on survival of antifolate treatment has been observed previously[184,186]. This mechanism was confirmed by methotrexate sensitivity assays in high folate medium, where a small but significant increase in the IC_{50} concentration for NF54 wild type parasites was observed. This shift was not seen in *PfΔmrp1* parasites, most likely because a maximal beneficial intraparasitic folate concentration was already reached. The fact that the increase in IC_{50} concentration for the *mrp1*-deleted line is much larger than for the folate supplemented parasites might be explained by the assumption that folate only partly reaches its intraparasitic target when added to the culture medium. A mechanism of resistance related to the folate concentration has been previously proposed in a study on *Pfmrp1* single nucleotide polymorphism selection in regions of prolonged sulfadoxine-pyrimethamine treatment[57]. Surprisingly, we did not observe decreased sensitivity towards the other anti-folate compounds. We speculate that the folate analogue, methotrexate, might have a different inhibitory mechanism than the other anti-folates.

Methotrexate does not only mimic folic acid in its DHFR binding capacity, it is also a competent substrate of human MRP transport proteins, including the folate exporters[163,164,187,188]. The differential transport of folate in *PfΔmrp1* parasites implies that also methotrexate is a likely substrate candidate for this export pump. This does not, however, explain the methotrexate-specific sensitivity shift in *PfΔmrp1* parasites, as deletion of the gene results in decreased export. In case methotrexate would indeed be a *PfMRP1* substrate, export from the cytosolic compartment of the parasite is abolished in *PfΔmrp1* parasites, which would increase intracellular methotrexate concentrations and could counteract folate containment effects.

In this study, we have identified folate as a likely *Pf*MRP1 substrate through untargeted metabolomics, and confirmed differential transport in *PfΔmrp1* parasites to the red blood cell compartment through LC/MS-MS. Drug sensitivity assays showed decreased sensitivity of *PfΔmrp1* parasites towards methotrexate. The involvement of *Pf*MRP1 in the efficiency of anti-folate malaria treatment is therefore anticipated.

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CHAPTER 4

Multidrug ABC transporters are essential for hepatic development of *Plasmodium* sporozoites

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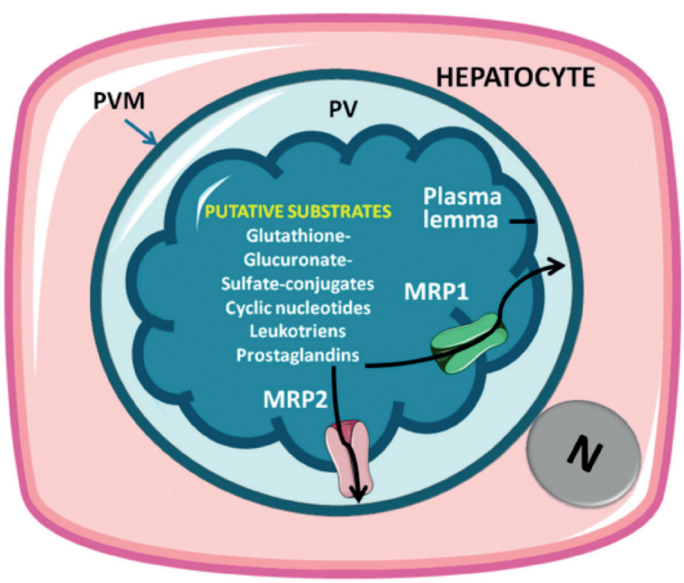
ABSTRACT

Multidrug resistance-associated proteins (MRP) belong to the C-family of ATP-binding cassette (ABC) transport proteins, and are known to transport a variety of physiologically important compounds and to be involved in the extrusion of pharmaceuticals. Rodent malaria parasites encode a single ABCC protein, whereas human parasites encode two: MRP1 and MRP2. Although associated with drug resistance, their biological function and substrates remain unknown. To elucidate the role of MRP throughout the parasite life cycle, *P. berghei* and *P. falciparum* mutants lacking MRP expression were generated. *P. berghei* mutants lacking expression of the single MRP as well as *P. falciparum* mutants lacking MRP1, MRP2 or both proteins have similar blood stage growth kinetics and drug-sensitivity profiles as wild type parasites. We show that MRP1-deficient parasites readily invade primary human hepatocytes and develop into mature liver stages. In contrast, both *P. falciparum* MRP2-deficient parasites and *P. berghei* mutants lacking MRP protein expression abort in mid- to late liver stage development, failing to produce mature liver stages. The combined *P. berghei* and *P. falciparum* data are the first demonstration of a critical role of an ABC transporter during *Plasmodium* liver stage development.



INTRODUCTION

ATP binding cassette (ABC) transport proteins are evolutionary well conserved membrane transporters that translocate structurally and functionally diverse compounds at the expense of ATP, even against steep concentration gradients[87]. The structure of a typical ABC transporter is composed of two transmembrane domains (TMDs), each consisting of six transmembrane helices (TM) and two cytosolic nucleotide binding domains (NBDs) **(Figure 1A)**[20]. Most of the ABC family members act as efflux systems and are therefore predominantly located on plasma membranes[87]. These transport proteins are well known for their role in multidrug resistance, as many classes of drugs are among their substrates. Mutations or upregulation of genes encoding ABC transporters may enhance efflux and decrease intracellular drug accumulation, ultimately resulting in resistance[14,191]. ABC proteins are classified into seven subfamilies (A-G), which can be distinguished upon phylogenetic analysis of the conserved NBDs and have differential compound specificities[148].

Proteins of the ABC transporter subfamily C (ABCC) are often referred to as the MRPs (multidrug resistance-associated proteins)[192]. The human MRP family consists of 13 members. These transporters predominantly translocate organic anions across the plasma membrane, including glutathione-, glucuronate- or sulfate- conjugates from endogenous and exogenous sources as well as cyclic nucleotides, leukotriens and prostaglandins that are involved in cellular signaling processes[193-195]. Several MRP proteins are known to transport drugs and are involved in resistance acquisition[196].

The genome of the human malaria parasite *Plasmodium falciparum* encodes sixteen ABC family members[148]. Three of these transporters, including the multidrug resistance protein 1 (*Pfmdr1*) which has been most extensively characterized[148], have indeed been associated with resistance to antimalarial drugs. The sequenced *Plasmodium* genomes contain either one or two genes encoding C-family ABC transport proteins. Human and non-human primate malaria parasites possess two genes, whereas rodent parasites have one gene (www.plasmodb.org)[148]. In *P. falciparum*, PF3D7_0112200 and PF3D7_1229100, encode the multidrug resistance protein 1 (*Pf*MRP1) and 2 (*Pf*MRP2), respectively. These proteins have 41% sequence identity at the amino acid level. Characterization by phylogenetic analysis of the highly conserved nucleotide binding domain (NBD), which consists of the Walker A, ABC signature and Walker B regions, showed

47.2% and 57.0% identity of the first and second NBD regions, respectively[17] (see **Figure 1A** for a schematic representation of the typical structure of ABCC proteins). The single *P. berghei mrp* gene (PBANKA_144380) has a syntenic genome location with *Pfmrp2* (www.plasmodb.org). DNA sequence alignment of PBANKA_144380 with *Pfmrp1* and *Pfmrp2* shows 36.7% and 42.0% identity, respectively. The higher sequence similarity of PBANKA_144380 in combination with its syntenic location with *Pfmrp2* suggests that it is the functional ortholog of *Pfmrp2*, as reported previously[148,197,] and is therefore referred to as *Pbmrp2* in this work.

Mutations in the gene encoding for *P. falciparum* MRP1 have been associated with increased resistance to antimalarials sulfadoxine-pyrimethamine, chloroquine and quinine, although its definitive role in drug responses remains controversial[11,57,198]. In addition, evidence has been presented that increased MRP2 expression confers tolerance to quinolone drugs[199] and the high genetic variability in *Pfmrp2* has been associated with reduced quinoline sensitivity[66]. We have previously shown that both MRP1 and MRP2 of *P. falciparum* are located on the plasma membrane of blood stage parasites[17], indicating that MRP-mediated transport may play a role in parasite growth within the erythrocyte. Previous analysis of a *P. falciparum mrp1* null-mutant, generated through targeted gene-deletion, provided evidence that blood stage MRP1-deficient parasites exhibited reduced growth; however, the ability to generate *mrp1*-null mutants demonstrates that MRP1 is dispensable for development within erythrocytes[64]. This may be the result of MRP2-mediated compensation of MRP1 function. In other organisms, MRP proteins are known to share many substrates and redundancy in transport function of different ABCC subfamily members has been reported[192]. Furthermore, a role of MRP1 in glutathione efflux and transport of different antimalarial drugs was demonstrated in this mrp1-null mutant[64]. Another study showed that the deletion of 4.1 kb in the *P. falciparum mrp2* upstream region resulted in altered transcription and increased expression, as well as increased tolerance to quinoline drugs[200].

The role of MRP transporters in alternative parasite life cycle stages has not been investigated. In particular their role in how the parasite establishes an infection in hepatocytes after an infectious mosquito bite. Sporozoites injected by a mosquito penetrate the microvasculature and traverse multiple hepatocytes before establishing a stable infection in the liver. The pre-erythrocytic phase is clinically silent, but represents a phase of high expansion, as a single sporozoite can give rise to thousands of merozoites. There is a large variation in the duration of the liver stage

between various *Plasmodium* species, as for *P. berghei* this is approximately 52 hours, whereas *P. falciparum* parasites require 6-7 days to reach full maturation. It is known that during the liver stage development the parasite regulates the host cell response and metabolism (e.g. inhibition of apoptosis, autophagy etc.) and this is known to play an important role in parasite survival inside the nucleated hepatocyte[201-203]. MRP proteins may play a role both in cellular signaling mechanisms and/or metabolism supporting development within the hepatocyte.

To further investigate the function and possible redundancy of MRP1 and MRP2 during all life cycle stages, and to determine their role in drug-sensitivity, we have genetically targeted the single *mrp* gene in the rodent parasite *P. berghei* and both *P. falciparum mrp* genes for gene-deletion. Analysis of the blood stages of the null-mutants of both species, including a *P. falciparum* mutant lacking both *mrp1* and *mrp2*, showed asexual blood stage development and drug-sensitivity profiles to a number of known antimalarial drugs similar to wild-type parasites. In contrast, liver stage analysis showed that MRP transport is essential for *Plasmodium* development in hepatocytes. We demonstrate that expression of *P. falciparum* MRP2 and its *P. berghei* ortholog at the plasma membrane of hepatocytic stages is critical for producing liver stage merozoites that can subsequently infect erythrocytes. Our study is the first to demonstrate a critical and specific role of a *Plasmodium* ABC transporter during liver stage development.

MATERIALS AND METHODS

Experimental animals and parasites

Female C57BL/6 and Swiss OF1 mice (6–8 weeks), Swiss albino CD-1 mice and BALB/c (4-6 weeks old) from Charles River were used. Four different reference parasite lines *P. berghei* ANKA were used. The wild type (WT) reference line cl15cy1[204] and three reference reporter lines that express fluorescent markers but are drug-selectable marker free: i) line 507cl1 (mutant RMgm-7, www.pberghei.eu) expressing GFP under the control of the eef1aa promoter ii) line 820cl1m1cl1 (WT Fluo-frmg; RMgm-164) expressing RFP under the control of the female gametocyte specific promoter lap4(ccp2) (PBANKA_131950) and GFP under the control of the male gametocyte specific promoter of PBANKA_041610 and iii) line 676m1cl1 (mutant RMgm-29; www.pberghei.eu) which expresses the fusion protein GFP-Luciferase under control of the *eef1α* promoter. NF54 was used as WT reference line for *P. falciparum*[205,206].

Generation and genotyping of gene-deletion mutants

The abcc (*mrp2*) gene (PBANKA_144380) was deleted in *P. berghei* using standard methods of transfection **(Fig 1A; Figure S1)**[207]. In short, a double cross-over strategy was applied using 5' and 3' target regions (TR) that were amplified from WT genomic DNA using primer pairs 2996/2993 and 2981/2982 **(Table S1)**. These products were cloned into the pL0001 plasmid (www.mr4.com), flanking the pyrimethamine resistant Toxoplasma gondii (Tg) dihydrofolate reductase-thymidylate synthase (*dhfr/ts*) selectable marker under the control of *P. berghei dhfr/ts* promoter. The resulting gene-deletion construct, pL1266, was linearized using the appropriate enzymes **(Table S1)**. Transfection and selection of transformed parasites with pyrimethamine was performed as described previously[204] using parasites of reference line 507cl1 and 820cl1 to obtain gene-deletion mutant lines 1025 and 1512, respectively. Clonal lines of the *PbΔmrp2* gene-deletion mutants (1025cl2, 1512cl1) were obtained by limiting dilution of the parasites in mice. Correct integration of the DNA construct was determined by Southern analysis of digested genomic DNA or chromosomes separated by pulse-field gel (PFG) electrophoresis. Southern blots were hybridized with *P. berghei* specific probes: 3'UTR *dhfr/ts* and 3'-region of *mrp2*. The last probe was PCR amplified from genomic WT DNA using primer pair 5094/5095 **(Table S1).** Transcription of *mrp2* was determined by Northern analysis of RNA obtained from WT and gene-deletion mutant blood stages of asynchronous *in vivo* infections or from synchronous blood infections of WT parasites[208]. Northern blots were hybridized with a 5'-UTR fragment of *mrp*, PCR amplified from genomic WT DNA using primer pair 5092/5093 and with the a/b-large subunit rrna probe as control (primer 644)[209.] Reverse transcriptase PCR reactions were carried out following manufacturer's recommendations (Invitrogen) with primer pair 136 and 138 which amplify a 1.3 kb fragment of the internal part of the open reading frame of *mrp2* **(Table S1)**. As a control for gDNA contamination, PCRs were carried out on cDNAs synthesized in the presence (+) or absence (-) of reverse transcriptase. No cDNA (-D) and *P. berghei* WT gDNA (G) were used as negative and positive controls, respectively.

For the generation of gene-deletion *P. falciparum* mutants, NF54 wild type parasites were cultured in a semi-automated culture system using standard in vitro culture conditions for *P. falciparum* and induction of gametocyte production was performed as previously described[153,205,210]. In short, human serum supplemented RPMI medium was changed twice daily and 0,5% hematocrit shaken cultures were maintained at parasitaemias below 20%. The *Pfmrp1* (PF3D7_0112200) and *Pfmrp2* (PF3D7_1229100) genes were deleted using a modified construct based on plasmid pHHT-FRT-

(GFP)-Pf52 for double crossover recombination through positive and negative selection **(Figure 1A; S2)**[151]. 5′ and 3′ TR were PCR-amplified (LA Taq, Takara) from genomic *P. falciparum* DNA of the NF54 strain using primer pair SR001 /SR0002 (*Pfmrp1* 5′ TR), SR003/SR004 for the *Pfmrp1* 3′ TR, SR005/SR006 for the *Pfmrp2* 5′ TR and SR007/SR008 for the *Pfmrp2* 3′ TR (Table S1). All PCR products were sequenced after TOPO TA (Invitrogen) sub-cloning, and consecutively cloned into pHHT-FRT-(GFP)-Pf52 upon digestion with *BssHII*/*BsiWI* and *XmaI*/NheI for the 5′ and 3′ TR, respectively, resulting in plasmids pHHT-FRT-(GFP)-*Pfmrp1* and pHHT-FRT-(GFP)-*Pfmrp2*. Transfection with these constructs and selection of mutant parasites was performed as described[211]. These lines were subsequently transfected with pMV-FLPe to remove the FRT flanked drug-selectable marker cassette as described[152], resulting in PfΔmrp1*FLPe (*PfΔmrp1*) and *PfΔmrp2*FLPe (*PfΔmrp2*) mutant lines **(Figure S2).** A consecutive gene deletion was made in the *PfΔmrp2* gene deletion mutant, targeting *mrp1* using plasmids pHHT-FRT-(GFP)-*Pfmrp1* as described above, thereby creating the double gene-deletion parasite *PfΔmrp1Δmrp2*. Clonal lines for the *PfΔmrp1*, *PfΔmrp2* and *PfΔmrp1Δmrp2* parasites were selected by limiting dilution in 96-well plates as described[212.] Genotype analysis of all gene deletion mutants was performed by an internal gene specific PCR using primer pair SR009/SR010 for *mrp1* and SR013/SR014 for *mrp2* **(Table S1)**, combined with an Expand Long range dNTPack (Roche) diagnostic long-range PCR (LR-PCR, using primer pairs SR011/SR012 for *mrp1* and SR015/SR016 for *mrp2*, restricted with the appropriate enzyme) **(Figure S2C)**. Northern analysis of RNA was performed as described above; blots were hybridized with the respective 5′ TR.

Generation and genotyping of transgenic *P. berghei* parasites expressing cmyc-tagged *Pbmrp2*

The pL1419 construct was used to generate transgenic *P. berghei* parasites expressing a C-terminally cmyc-tagged MRP2[213]. The smac targeting region was replaced by a PCR-amplified targeting region of *mrp2* using primer pair 5090/5091 **(Table S1)**. Subsequently, the mCherry reporter cassette was replaced in this construct with a DNA-fragment containing the triple cmyc cassette which was PCR-amplified from plasmid pL1435 using primer pair 4816/4817 **(Table S1)** resulting in the final construct pL1672. After linearization with restriction enzyme *NdeI*, this construct was used to transfect parasites of line 676cl1 as described above. Transgenic parasites were selected and cloned as described above (*mrp::cmyc,* 1732cl3). Correct integration of the DNA construct was determined by Southern analysis of chromosomes separated by PFG electrophoresis hybridized with the *P. berghei* specific probe 3′UTR *dhfr/ts*.

Phenotypic analysis of *P. berghei* gene-deletion mutants

The *in vivo* multiplication rate of asexual blood stages was determined during the cloning procedure as described[214]. Parasitaemias (in percentage) in Swiss OF1 mice injected with a single parasite were determined at day 8 to 11 on Giemsa stained blood films. Per mouse, an estimated number of 1.2 x 1010 erythrocytes (2 mL of blood) was used to calculate the multiplication rate per 24 hours. The percentage of infected erythrocytes in mice infected with reference lines of the *P. berghei* ANKA strain consistently ranged between 0.5-2% at day 8 after infection, resulting in a mean multiplication rate of 10 per 24h[204]. In addition, parasite growth was analyzed in Swiss mice (5 mice per group) infected intraveneously (i.v.) with 200 parasites of two reference lines (WT and 507cl1) and two *mrp2* gene-deletion mutants (*PbΔmrp2*-a, 1025cl2 and *PbΔmrp2*-b, 1512cl1). In these mice, parasitemia was determined in Giemsa-stained blood smears on days 1–7 after infection.

Feeding of *A. stephensi* mosquitoes with *P. berghei* infected mice and sporozoite collection from salivary glands as well as *P. berghei* gliding motility were performed as described[215]. Infectivity of *P. berghei* sporozoites and development was determined in cultures of Huh7 cells[215]. For analysis of liver stage development by immunofluorescence, parasites were stained with the following primary antibodies: anti-PbEXP1 (PBANKA_092670) raised in chicken[216], anti-PbHSP70 (PBANKA_081890) raised in mouse[217] and anti-MSP1 (PBANKA_083100) antibody obtained through MR4 (MRA-78, www.mr4.org)[218].

C57BL/6 and Swiss mice were i.v. inoculated with $5x10^4$ and $3x10^5$ sporozoites. Blood stage infections were monitored by Giemsa-stained thin smears collected on day 4–14 post-inoculation. The prepatent period (measured in days) was defined as the first day with a parasitemia of 0.5–2%.

Phenotypic analysis of *P. falciparum* gene-deletion mutants

P. falciparum blood stage multiplication was evaluated upon subculturing at 0,1% parasitaemia in a 2,5% hematocrit candle-jar culture with daily change of medium. Parasitaemias were determined on Giemsa-stained blood films of day 0, 3, 5, 6 and 7. Mature stage V gametocytes were produced in cultures optimized for gametocytogenesis as described previously[206]. Gametocytaemia and gametocyte morphology were evaluated after 14 days.

Drug sensitivity assays for *P. falciparum* parasites were performed using an adapted *Plasmodium* lactate dehydrogenase (pLDH) activity assay employing resazurin conversion as a read-out[154]. Cultures were started in 100 µL sample format at 1% parasitaemia and 2,5% hematocrit and incubated for 72 hours. Antimalarial sensitivity was tested for chloroquine, artemisinin, dihydroartemisinin, atovaquone, lumefantrine, quinine and mefloquine, all purchased from Sigma-Aldrich. Each compound concentration was tested in triplicate and in three independent assays. Resorufin concentration was measured with a Synergy 2 (Bio-Tek, USA) fluorescent plate reader at 530 nm excitation and 590 nm emission. Solvent exposed parasite samples represented 100% growth, and samples with maximal inhibitory concentrations were equalized to 0% growth. The inhibition curve fitting and statistical analysis of IC_{50} concentrations were performed in GraphPad Prism, version 5.03. An inhibitor response (variable slope) fit was used to determine inhibitory curves and hill slopes were adjusted to NF54 steepness. Statistical analysis was performed in GraphPad where comparisons for significant differences between the obtained log IC_{50}-values of wild type and mutant groups were carried out by a one-way-Anova.

Male exflagellation capacity was evaluated after stimulation with fetal calf serum at pH 8.0[152]. In the standard membrane feeding assays, oocyst development was monitored at day 7 and selection of sporozoites from mosquito salivary glands was performed around day 14[153,219]. Sporozoite gliding motility on glass plates was evaluated by visualizing gliding trails using anti-CSP (PF3D7_0304600; 3SP2) antibody and hepatocyte traversal capacity was analyzed through FACS analysis of rhodamine-labelled dextran positive cells, as previously described[220,221]. The infection and development monitoring of *P. falciparum* parasites in primary human hepatocytes that were freshly isolated from human remnant material after tumor removal surgery were performed during maximal ten days as described previously[222,223]. Immunocytochemistry was performed after fixing the cultures with 4% para-formaldehyde (PFA), quenching with 0.1 M glycine, blocking with 10% FCS and permeabilization with 1% Triton-X-100. Localization of was performed using specific antibodies against HSP70 (PF3D7_0930300) raised in rabbit[224]; CSP raised in mouse[225]; EXP1 (PF3D7_1121600) raised in mouse[226], MSP1 (PF3D7_0930300) raised in mouse[227], MRP1 and MRP2, both raised in rabbit[17]. Epitopes were then identified using anti-rabbit-ALEXA488 and anti-mouse-ALEXA594 (Molecular probes) and nuclei were stained with 1 µg/ml DAPI. Analysis was performed using a Olympus FV1000 and a Zeiss LSM510 meta microscope.

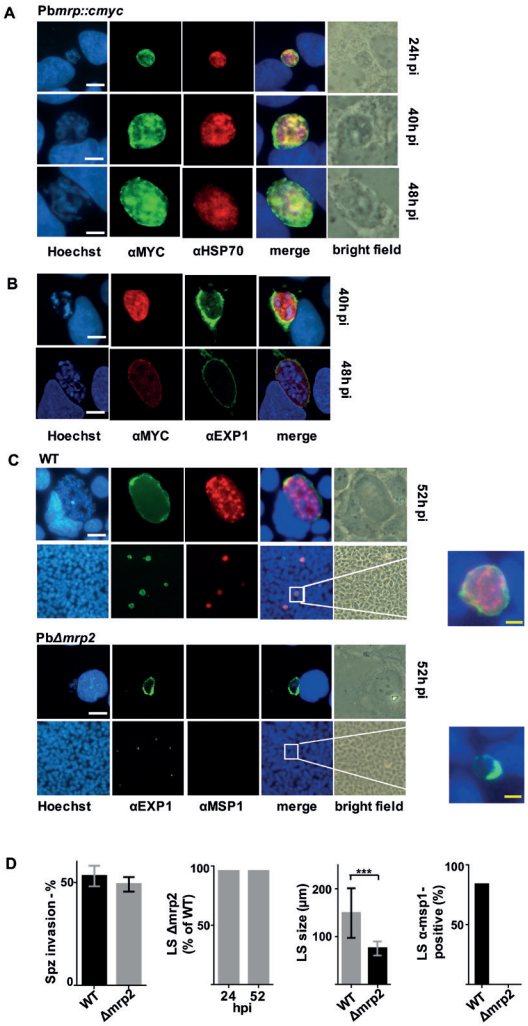


Figure 2. Liver stage development of *P. berghei Pbmrp::cmyc* and *PbΔmrp2* parasites

A Development of *Pbmrp::cmyc* liver stages in cultured hepatocytes visualized by staining with antibodies recognizing cmyc (green) and the parasite cytoplasm (anti-HSP70; red). Nuclei are stained with Hoechst-33342. Scale bar 10μm. **B** Standard (top row) and confocal (bottom row) fluorescence microscopy images of *Pbmrp::cmyc* liver stages in cultured hepatocytes. *Pbmrp::cmyc* liver stages were stained with antibodies recognizing cmyc (red) and the parasite vacuole membrane protein EXP1 (green). Nuclei are stained with Hoechst-33342. Images were acquired with a DM RA Leica fluorescence microscope and confocal microscope Leica TCS SP8 X with white light laser (WLL). Scale bar 10μm. **C** WT and *PbΔmrp2* liver stages in cultured hepatocytes visualized by staining with antibodies recognizing the PVM (anti-EXP1; green) and the formation of merozoites (anti-PbMSP1, red). Hoechst-33342: nuclear staining (blue). Scale bar 10 μm. **D** Development of liver stages in cultured hepatocytes at different hours post infection (hpi) of wild type (WT) parasite (left panel) and *mrp2* gene-deletion mutant (*PbΔmrp2*). Both WT and *PbΔmrp2* express the GFP reporter protein under control of the constitutive *eef1a* promoter. Fluorescence images are from live parasites. Nuclei are stained with Hoechst-33342 (blue). Scale bar 10μm. Parasites appear to invade and develop like WT parasites until 30 hpi; they become significantly smaller from 40 hpi (see B) and contain much fewer nuclei than WT parasites at 52 hpi. **E** Sizes (area of fluorescence) of WT and *PbΔmrp2* liver stages in cultured hepatocytes at 24, 30, 40, 48 and 52 hpi. **F** Invasion of hepatocytes by sporozoites and development of liver stages (LS) WT and *PbΔmrp2* parasites. LS development is determined by counting liver stages at 24 and 52 hours post invasion (hpi), the size of LS at 52 hpi and the percentage of LS that stain with anti MSP1-antibodies at 52 hpi.

Immunisation of mice with mutant (*PbΔmrp2*) *P. berghei* sporozoites

P. berghei sporozoites were collected at day 21-27 after mosquito infection by dissection of salivary glands. 5 BALB/c mice per group were immunized by intravenous injection with either saline, 400, 800 or 1200 PbΔmrp2 sporozoites. Immunized mice were challenged after 21 days by intravenous injection of 1x104 sporozoites of the *P. berghei* ANKA reference line 676m1cl1 which expresses the GFP-Luciferase reporter protein. Liver stage development in the challenged mice was monitored by real time *in vivo* imaging and parasite load was quantified by measuring luciferase activity using a Lumina luminescence detector (Caliper Life Sciences, USA)[228]. After challenge, mice were monitored for blood infections by analysis of Giemsa stained films of tail blood at day 4-21. Pre-patency (measured in days after sporozoite inoculation) was defined as the day when a parasitemia of 0.5-2% was observed.

Ethics Statement

All animal experiments performed at the LUMC were approved by the Animal Experiments Committee of the Leiden University Medical Center (DEC 07171; DEC 10099) and all animal experiments performed at the RUNMC were approved by the Animal Experiments Committee of the Radboud University Nijmegen Medical Center (DEC 2010-229). The Dutch Experiments on Animal Act is established under European guidelines (EU directive no. 86/609/EEC regarding the Protection of Animals used for Experimental and Other Scientific Purposes). All mice procedures conducted at the UPR-School of Medicine were approved by the IACUC of the Medical Sciences Campus, University of Puerto Rico, and all work was done in accordance with the "8th Guide for the Care and Use of Laboratory Animals" (National-Research-Council, 2011) and regulations of the PHS Policy on Humane Care and Use of Laboratory Animals.

Primary human liver cells were freshly isolated from remnant material upon tumor removal surgery. As samples were anonymized, no diagnostic techniques were applied and material was destroyed upon use, general approval for use of remnant material prior to surgery was sufficient according to Dutch ethical legislation as described in the Medical Research (Human Subjects) Act, and confirmed by the Committee on Research Involving Human Subjects region Arnhem-Nijmegen.

RESULTS

The single *P. berghei* MRP protein, MRP2, is dispensable for asexual blood stages

We found evidence for expression of *Pbmrp2* in blood stages by Northern analysis. In blood

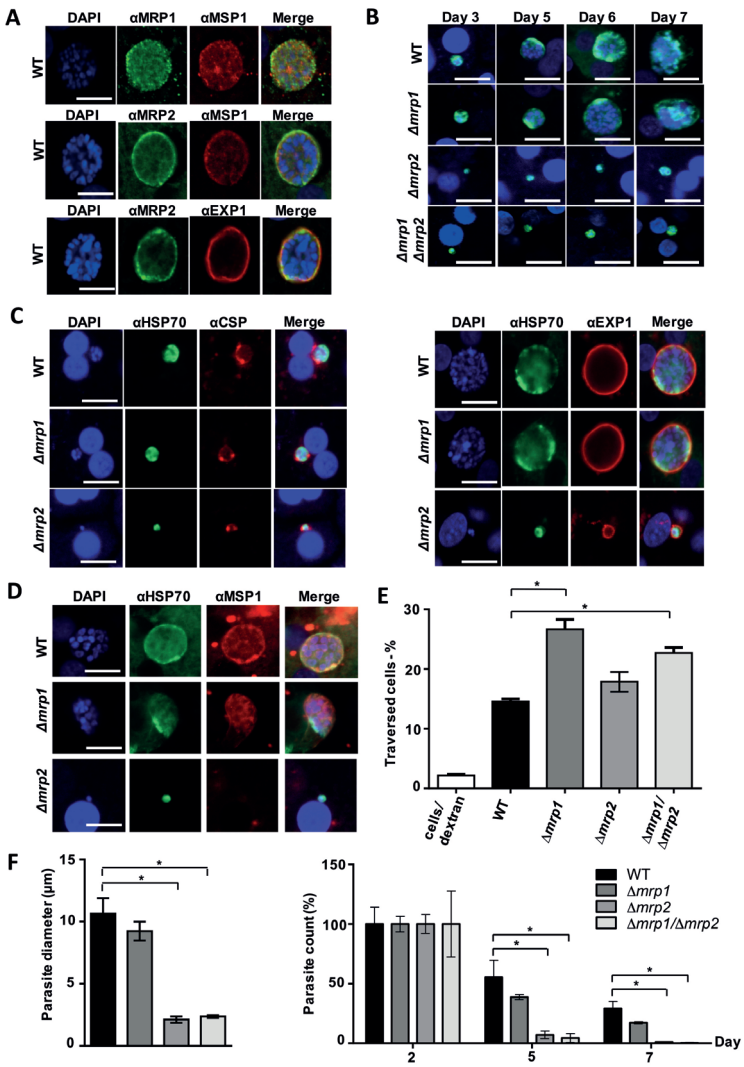


Figure 3. Liver stage development of *P. falciparum PfΔmrp* parasites in human primary hepatocytes
A Localization of different proteins in WT liver stages (LS) visualized by staining antibodies recognizing MRP1, MRP2 (green), MSP1 (merozoite formation; red) and EXP1 (PVM; red). DAPI: nuclear staining (blue). Scale bar 10 µm. **B** Development of WT and mutant LS at different days post infection, visualized by staining of the cytoplasm with anti-HSP70 antibodies (green). DAPI: nuclear staining (blue). Scale bar 10 µm. **C** Localization of circumsporozoite protein (CSP; red), EXP1 (PVM; red) and HSP70 (cytoplasm; green) in LS of WT and mutant parasites by staining with antibodies. DAPI: nuclear staining (blue). Scale bar 10 µm. **D** Localization of MSP1 (merozoite formation; red) and HSP70 (cytoplasm; green) in LS of WT and mutant parasites by staining with antibodies. DAPI: nuclear staining (blue). Scale bar 10 µm. **E** Hepatocyte cell traversal of WT and mutant sporozoites as determined by FACS counting of dextran positive HCO4 cells. The mean percentage of traversed cells was for WT 14.6% (95% CI 13.4-15.8), for *PfΔmrp1* 26.7% (95% CI 19.6-33.7), for *PfΔmrp2* 17.9% (95% CI 10.8-25.0) and for *PfΔmrp1Δmrp2* 22.7% (95% CI 18.8-26.6). Dextran control: hepatocytes in the presence of Dextran but without addition of sporozoites. F Size and numbers of WT and mutant LS in cultures of human primary hepatocytes. The size was determined at day 6 post infection (p.i.). Parasite numbers per well were counted at different days p.i.

stages of wild type (WT) parasites, Northern analysis showed two different-sized transcripts of approximately 7.5 and 8 kb, indicating alternative processing of the untranslated regions as in the *Pbmrp2* gene no introns are present **(Figure 1B)**. The presence of *Pbmrp2* transcripts have been reported previously in blood stage parasites; however, the protein has not been detected in published proteome analyses of *P. berghei* blood stages (www.plasmodb.org). To analyze whether the MRP2 protein is expressed in blood stages we generated a transgenic line expressing a cmyc-tagged version of *Pb*MRP2 (*mrp*::*cmyc*) using a DNA construct that was integrated by single cross-over into the endogenous *Pbmrp2* locus, resulting in a C-terminally 3xcmyc-tagged MRP2 **(Figure S1)**. The *mrp*::cmyc parasites showed *in vivo* asexual multiplication rates during the initial phase of infection similar to wild type (mean multiplication rate/24 hour (s.d.): WT 10 (0), n=12; *mrp*::cmyc 10 (0), n=3). We were unable to detect *Pbmrp::cmyc* protein in blood stages by immunofluorescence using anti-cmyc antibodies, whereas the tagged protein was readily detected in liver stages (see below). The absence of *Pbmrp::cmyc* in blood stages indicates that either *Pb*MRP is not expressed as a protein in blood stage parasites or that the expression level is too low for detection by immunofluorescence. To investigate if *Pb*MRP2 was essential for growth within erythrocytes, *Pb*MRP2 null-mutants were generated. The *Pbmrp2* gene was targeted for deletion by double cross-over homologous integration of a DNA construct containing a *dhfr/ts* selection marker using standard methods of genetic modification[204] **(Figure 1A, S1).** In two independent experiments, gene-deletion mutants (*PbΔmrp2*-a and *PbΔmrp2*-b) were selected indicating that *Pb*MRP2 is not essential for asexual blood stage development. Correct deletion of *Pbmrp2* was confirmed through Southern integration analysis and diagnostic PCR (Figure S1) as well as Northern and RT-PCR analysis **(Figure 1B, S1).** The *PbΔmrp2* parasites showed wild type (WT) *in vivo* asexual multiplication rates through the course of an infection **(Figure 1C)** and during the initial phase of infection after mice had been infected with a single parasite (mean multiplication rate/24 hour (s.d.): WT 10 (0), n=18; *PbΔmrp2*-a 10 (0) n=7; *PbΔmrp2*-b 10 (0) n=5).

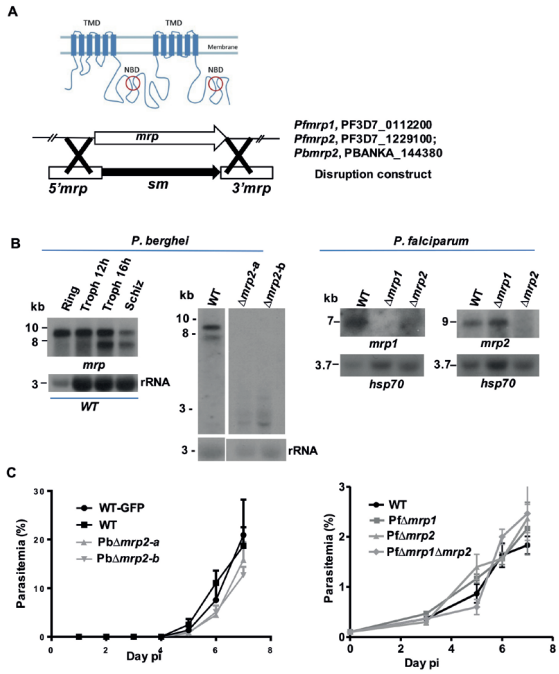


Figure 1. Generation and characterization of *P. berghei* and *P. falciparum* mutants lacking expression of MRP transporters

A Schematic representations of a typical MRP transporter (adapted from[17], with permission) and DNA constructs to disrupt the *mrp* genes. Shown are the transmembrane domains (TMD) with 6 transmembrane segments and the nucleotide binding domains (NBD), the DNA constructs used to delete the complete *mrp* genes by double cross-over homologous recombination using the 5' and 3'UTR regions replacing the gene with a drug-selectable marker cassette (SM). See Figures S1 and S2 for details of the gene-deletion constructs and genotype analysis of the different *P. berghei* and *P. falciparum* mutants. **B** Northern analysis of *mrp* transcripts in wild type (WT) and mutant *P. berghei* and *P. falciparum* parasites confirms absence of transcription of the *mrp* genes in blood stages of the mutant parasites. *P. berghei* (left panel): transcripts detected in WT blood stages. *P. berghei* (right panel): absence of *mrp* transcripts in mutant parasites. Northern blots were hybridized with a probe specific for the 5'UTR of *mrp2* and as a loading control with probe L644R recognizing large subunit ribosomal RNA (rRNA). *P. falciparum* (upper panel): Northern analysis of *mrp1* and *mrp2* transcripts in WT and mutant parasites. Blots were hybridized with probes specific for the 5'UTR of *mrp1* and *mrp2* and as a loading control with a probe recognizing *hsp70* transcript (gene ID PF3D7_0818900 lower panel). **C** Asexual multiplication of WT and mutant parasites. Left: Parasitemia in mice infected with *P. berghei* WT (WT and WT-GFP) and *PbΔmrp2* parasites. Right: Parasitemia in candle jar cultures of *P. falciparum* WT and the different *PfΔmrp* mutants. Parasitaemia was determined in Giemsa-stained thin blood smears.

MRP protein expression is dispensable for *P. falciparum* blood stage growth in vitro

In contrast to *P. berghei*, expression of the two MRP proteins in *P. falciparum* blood stages was previously demonstrated both by transcriptome and proteome analyses (www.plasmodb.org), as well as by immunofluorescence assays using MRP1- and MRP2-specific antibodies[17]. We generated three different gene-deletion mutants by standard reverse genetic approaches: one lacking *Pfmrp1* (*PfΔmrp1*), another lacking *Pfmrp2* (*PfΔmrp2*) and a double gene-deletion mutant lacking both *Pfm-*

rp1 and *Pfmrp2* (*PfΔmrp1Δmrp2*). *PfΔmrp1* and *PfΔmrp2* were generated by gene-deletion through double cross-over homologous recombination where the genes were replaced with the hdhfr::gfp selectable marker cassette **(Figure S2)**. After deletion of the *mrp* genes, the selectable marker was removed from the genome through FLPe-mediated excision of the sequence between the FRT sites integrated in the gene-targeting construct **(Figure S2)**, resulting in the selectable-marker free mutants *PfΔmrp1* and *PfΔmrp2*[152]. The double gene-deletion mutant *PfΔmrp1Δmrp2* was subsequently generated by targeting *Pfmrp1* in the selection-marker free *PfΔmrp2* mutant using the DNA construct used for generation of *PfΔmrp1* **(Figure S2)**. Diagnostic PCR analysis in combination with Northern analysis of transcription confirmed correct deletion of the *mrp* genes in clonal lines of the three different mutants **(Figure 1B** and **Figure S2)**.

Analyses of asexual blood stage parasites in vitro showed that asexual blood stage multiplication in candle jar culture conditions for all three mutants were comparable to parent WT NF54 parasites of the *P. falciparum* NF54 strain **(Figure 1C)**. Also, during routine culturing in the semi-automated culture system, growth rates comparable to WT were observed for all three mutant lines and parasitaemias of >20% were readily obtained in more than 5 independent cultures per mutant. In addition, gametocyte production of the three mutants was not significantly different from WT **(Table 1)**. Morphology by light-microscopy was comparable to WT gametocytes and male gametocytes of mutants successfully produced exflagellation centers upon activation of gametogenesis **(Table 1)**. Combined, these observations demonstrate that expression of MRP proteins is dispensable for *P. falciparum* development of asexual and sexual blood stages *in vitro*.

Parasite	Gametocytes (106 per mL; mean and range)	Exfl[1]	Oocyst production[2]	% Infected mosquitoes (mean and 95% CI)	Sporozoite number per oocyst (mean and range)	% of sporozoites with ≥1 gliding trail - (mean and range)
WT	1.9 (1.12-2.8)	++	21.5 (13.1-29.9)	93.9 (89.9-97.8)	2850 (2388-3538)	30.2 (28.2-33.3)
Δmrp1	2.2 (0.8-3.2)	++	13.2 (-7.9-34.3)	95.0 (79.1-111)	3982 (3569-4360)	53.6 (31.6-70.0)
Δmrp2	1.6 (0.6-2.8)	++	35.0 (23.3-46.7)	90.0 (71.6-108)	3152	66.0 (57.1-70.8)
Δmrp1Δmrp2	0.9 (0.3-1.6)	++	3.7 (0.75-6.57)*	46.9 (25.3-68.4)*	3517 (1614-5421)	ND

Table 1: Gametocyte production and mosquito development of *P. falciparum PfΔmrp* parasites

[1] Exflagellation (Exfl) of male gametocytes was determined in stimulated samples from day 14 gametocyte cultures in wet mounted preparations at 400x magnification using a light microscope; ++ score = >5 exflagellation centers per microscope field

[2] Oocyst production is the median (95% CI) of the oocysts counted at day 7 after feeding of the mosquitoes, analyzed in 130, 40, 40 and 220 mosquitoes for the respective parasite strains.

* Significant different from WT (p<0. 05); One-way ANOVA

The absence of MRP expression in blood stages does not affect sensitivity of *P. falciparum* to a number of known anti-malarial drugs

To determine if MRP contributes to drug sensitivity, in vitro sensitivity of *PfΔmrp1*, *PfΔmrp2* and *PfΔmrp1Δmrp2* to a number of known anti-malarial was assayed. The IC_{50} concentrations of 6 out of 7 drugs tested (chloroquine, artemisinin, dihydroartemisinin, atovaquone, lumefantrine, quinine) were not significantly different from WT **(Figure S3, Table S2)** and were comparable to previously published data[175,229-236]. Only for mefloquine we detected a small but significant decrease in sensitivity for the three mutants, however all IC_{50} values are well above previously reported values.

***P. berghei* MRP2 is essential for complete development within hepatocytes**

Whereas no published evidence for expression of MRP transporters is available for the liver stages of *P. berghei* and *P. falciparum*, RNA sequencing analysis in *P. yoelii* reveals that it is transcribed at this stage of parasite development (www.plasmodb.org). Moreover, and in agreement with this, we can readily detect *Pbmrp::cmyc* protein expression during liver stage development by immunofluorescence analysis of infected hepatocytes *in vitro* at 24, 40, 48 and 52h **(Figure 2A, Figure S4A and B).** *Pbmrp::cmyc* showed a circumferential pattern when compared to the cytoplasmic HSP70 **(Figure 2A)** protein and did not co-localize with EXP1, a protein on the parasitophorous vacuole membrane (PVM) **(Figure 2B, Figure S4A and B).** These staining patterns suggest that *Pb*MRP2 is at the plasma lemma membrane of liver stage parasites. The transgenic parasite expressing MRP::cmyc showed a normal development of liver stages *in vitro* and *in vivo* **(Figure S5)**. As the transgenic parasites exhibit a WT developmental phenotype (unlike the MRP deletion mutants) both in mice and mosquitoes we believe that tagging *P. berghei* MRP did not alter its function or its intracellular location.

To assess if deletion of MRP affected parasite transmission, *PbΔmrp2*-infected mosquitoes were allowed to feed on naïve mice. None of the mice (nr=5) developed a blood stage infection (data not shown). Similarly, intravenous injection of $5x10^4$-$3x10^5$ purified *PbΔmrp2* salivary gland sporozoites (2 experiments; 14 C57Bl/6 mice) did not result in a blood stage infection. Gliding motility and in vitro hepatocyte invasion of *PbΔmrp2* sporozoites were comparable to WT parasites **(Figure 2)**. Gliding motility assays showed that 70.2% (+7.3) of the *PbΔmrp*2 sporozoites produced the characteristic circular trails compared to 73.5% (+4.2) of WT sporozoites. Infection of Huh7 cell cultures with *PbΔmrp2* and WT sporozoites show that 49.1% (+3.5) and 53.1% (+5) of the sporozoites were able

A: DNA-constructs

Gene	Construct	Basic construct	Primer	Sequences	Restriction sites	Description
Pfmrp1	PFC-MRP1	PHHT-FRT-FCU	SR001	GGGGCGCGCGAATCA AAAGGAGGTTCTTC	*BsHII*	5'- mrp1 targeting region F; probe
			SR002	GGGCGTACGTTCATG TAATACGCATACC	*BsiWI*	5'- mrp1 targeting region R; probe
			SR003	GGGCCCGGGCTTAC ACACACCCATGCATAC	*XmaI*	3'- mrp1 targeting region F
			SR004	GGGGCTAGCGTAAC TATTTCTGACCAATTC	*NheI*	3'- mrp1 targeting region R
Pfmrp2	PFC-MRP2	PHHT-FRT-FCU	SR005	GGGGCGCGCCTTCT TACATTTGTTTATCG	*BsHII*	5'- mrp2 targeting region F; probe
			SR006	GGGCGTACGCGAAA TTGTAAACGCTTCTCCG	*BsiWI*	5'- mrp2 targeting region R; probe
			SR007	GGGCCCGGGCCGA ATTAGCTAACTTGC	*XmaI*	3'- mrp2 targeting region F
			SR008	GGGGCTAGCGGTTC ATGCAAATGTTTATGC	*NheI*	3'- mrp2 targeting region R
Pbmrp2	pL1266	pL0001	2996	GGGGGTACCGATGAA CCGAAAAATGAAGAA AAATTATATAGATAAG	*KpnI*	5'- mrp2 targeting region F
			2993	GGGAAGCTTGTTTTG CAGTTTTAGATTGTAAT AATGGAG	*HindIII*	5'- mrp2 targeting region R
			2981	GGGGGGGGATCCTATT GAAGGAATTGAAACTT TTACACCATA	*BamHI*	3'- mrp2 targeting region F
			2982	GGGGGGTCTAGAAAA TACTTCTGTGTGAATTGC CTTTGCTGA	*XbaI*	3'- mrp2 targeting region R
	pL1672	pL1419	5090	GGGACTAGTCATTTT ATAGTCATAATATAGGG	*SpeI*	3'- mrp2 targeting region F
			5091	GGGGGATCCATTTGCC TGGTCCTGTATAATATTAG	*BamHI*	3'- mrp2 targeting region R (without stop codon)
		pL1435	4816	CGGGATCCGGTGGAGGTG GAAGATCCGAACAAAAAC TCATCTCAGA	*BamHI*	3xcmyc-3'UTR cam F
			4817	CGATATCGAATTCGAG CTCGGTACC	*EcoRI*	3xcmyc-3'UTR cam R

to invade hepatocytes, respectively. During the first 24-30 h the development of *PbΔmrp2* liver stages with respect to number, size and number of nuclei per parasite was similar to WT liver stage development. In contrast, subsequent intra-hepatocytic development of *PbΔmrp2* parasites was abnormal while parasites appear to invade and develop like WT parasites until 30 hpi they become significantly smaller from 40 hpi and contain fewer nuclei than WT parasites at 52 hpi **(Figure 2C,**

D and E). While expression of the PVM protein EXP1 was readily detected in *PbΔmrp2* parasites, the liver stages remained MSP1-negative during the complete culture period. The combined observations demonstrate that *PbΔmrp2* parasites can invade hepatocytes, establish a PVM and start nuclear division but are unable to form mature schizonts containing infectious merozoites.

***P. falciparum* MRP2 is essential for development of mature liver schizonts**

Expression of both MRP proteins in *P. falciparum* parasites was analyzed in cultured primary human hepatocytes using specific antibodies against both proteins[17]. A circumferential staining pattern was observed for both *Pf*MRP1 and *Pf*MRP2, indicating they are likely present in the plasma lemma **(Figure 3A, Figure S4C),** which is in agreement with previous observations of *Pf*MRP1 and *Pf*MRP2 localization in *P. falciparum* blood stages[148]. Also co-localization of the MRP proteins with plasma membrane protein MSP1[237] **(Figure 3A)** on membranes separating individual merozoites at day 6 indicated a plasma lemma localization. However, staining of the PVM-resident protein EXP1 also shows a tight circumferential staining pattern in *P. falciparum* liver stages from cultured primary hepatocytes that makes it difficult to determine a conclusive MRP localization in *P. falciparum* infected hepatocytes.

Parasites of all three mutants produced oocyst and sporozoites in A. stephensi mosquitoes. The two single gene-deletion mutants, *PfΔmrp1* and *PfΔmrp2*, produced oocyst numbers in the same range as WT, whereas oocyst production by the *PfΔmrp1Δmrp2* double gene-deletion mutant was strongly reduced **(Table 1)**. Sporozoite production per oocyst in all three mutants was not significantly different from WT **(Table 1).** Sporozoites of the *PfΔmrp1* and *PfΔmrp2* clones showed effective gliding motility as well as hepatocyte traversal **(Figure 3E)**. *PfΔmrp1* and *PfΔmrp1Δmrp2* sporozoites showed even increased traversal rates compared to WT. These combined observations show that all mutants produce viable sporozoites that are able to glide and traverse hepatocytes.

In primary human hepatocytes at day 2 p.i., a mean of 107 infected hepatocytes per well (95% CI 42-172) was observed for NF54 WT parasites, and for the 3 mutant lines, *PfΔmrp1*, *PfΔmrp2*, and *PfΔmrp1Δmrp2*, 448 (95% CI 375 - 521), 348 (95% CI 249-417) and 343 (95% CI 195-492), respectively **(Table S3)**. The development of liver stages of *PfΔmrp1* was similar to that of WT parasites, both in total numbers, size and morphology **(Figure 3B-F; Table S3)**. Both WT and *PfΔmrp1* showed a gradual decrease in parasite numbers between day 2 and 6, as previously shown for WT parasites[238]. A strong increase in parasite diameter and nucleic content was observed in WT and *PfΔmrp1* parasites **(Figure 3B, F; Table S3)**. In

both WT and *PfΔmrp1*, multi-nucleated parasites were already observed on day 2 p.i.; the number of nuclei per parasite increased rapidly up to day 7 **(Figure 3B)**. In strong contrast, liver stage development of both *PfΔmrp2* and *PfΔmrp1Δmrp2* parasites was aberrant compared to WT in terms of numbers, size, and morphology **(Figure 3F; Table S3)**. After 7 days of intra-hepatocyte development, these parasites measured diameters comparable to WT day 2 p.i.. In addition, nuclear division was strongly inhibited in parasites of both lines. On day 2 p.i. all parasites showed only a single, DAPI-positive, nucleus; on day 7 p.i. a maximum number of only 6 nuclei was observed.

Analysis of liver stages using antibodies against CSP and EXP1 was used to determine if these proteins were expressed in all three mutants. CSP[40] was observed in both WT and mutant parasites at day 2-5 p.i. **(Figure 3C)**.EXP1 shows the typical circumferential staining pattern in all mutants, indicating that the lack of MRP1 and/or MRP2 expression does not affect PVM formation **(Figure 3C).** In contrast, MSP1 expression could not be detected in *PfΔmrp2* and *PfΔmrp1Δmrp2*, whereas expression was readily observed in both WT and *PfΔmrp1* parasites **(Figure 3D)**. The lack of MSP1 expression indicated the absence of merozoite formation in *PfΔmrp2* and *PfΔmrp1Δmrp2* mutants[239]. The phenotype of arrested liver stage development of the *P. falciparum* mutants lacking *Pf*MRP2 is similar to the phenotype of *P. berghei* mutants lacking *Pb*MRP2, demonstrating that these proteins play an essential role in development of liver stages and formation of infectious merozoites.

The abortion of liver stage development during mid-liver stages and the complete attenuation of *PbΔmrp* parasites are both features that can make such mutants attractive candidates for whole parasite vaccination strategies using attenuated sporozoites[240]. In preliminary experiments we evaluated protection induced by immunization of BALB/c mice with low doses of *PbΔmrp2* sporozoites. 5 mice per group were immunized with a single dose of 1200, 800 and 400 sporozoites. These immunized mice were challenged after 21 days by intravenous injection of sporozoites from the *P. berghei* ANKA reference line 676m1cl1, which expresses the GFP-Luciferase reporter protein. Liver stage development and parasite load were monitored in the challenged mice by real time *in vivo* imaging, which showed the absence of detectable liver loads in 80-100% of the immunized mice **(Figure S6)**. All control mice showed high liver loads and developed blood stage parasitemia with a prepatent period of 5 days. In contrast, only one immunized mouse (dose of 800 sporozoites) had a liver load and two mice in this group developed blood stage parasitemia whereas all other immunized mice did not develop parasitemia (up to 21 days after challenge). These observations

demonstrate that single immunisation with doses as low as 400 sporozoites can induce sterile protective immune responses.

DISCUSSION

Parasites interacting with the host environment require cellular mechanisms for either molecular exchange with or defense against host cell metabolites. Energy-dependent transporters, such as ABC proteins, are responsible for maintaining this metabolic homeostasis. Identifying which transporters are essential for parasite development, transmission or metabolite efflux in *Plasmodium* spp. will provide critical information about their biological role. In this study, the role of MRP in the *Plasmodium* life cycle was investigated by the analysis of gene-deletion mutants that lack expression of these proteins. We demonstrate that in both *Plasmodium* species, MRP proteins are dispensable for growth within erythrocytes and for sporogonic development in the mosquito. In contrast, we observed an essential function of MRP2 for development in hepatocytes for both *Plasmodium* species. Whereas MRP2-deficient parasites readily invade hepatocytes at WT rates and establish a PV as shown by the expression and correct localization of EXP1, they are unable to mature into liver stage schizonts as shown by the reduced size, DNA replication and the absence of MSP1, a marker for merozoite formation[239]. The lack of MRP2 alone resulted in complete developmental arrest of liver stages, while the phenotype of the *mrp1* gene deletion mutant was not altered in any of the *P. falciparum* life cycle stages. This indicates that MRP2 transports essential substrates during liver stage development, a function that cannot be compensated for by MRP1 or other (ABC)-transporters.

Compared to many other organisms including humans, malaria parasites contain fewer genes belonging to the ABC transporters of subfamily C. Rodent *Plasmodium* species encode only a single MRP protein, whereas in human parasites two members have been identified. The single copy abcc gene of *P. berghei* is the syntenic ortholog of *Pfmrp2* and shows a higher sequence similarity to *Pfmrp2* compared to *Pfmrp1*, which is suggestive of functional orthology of the *P. berghei* MRP protein and *Pf*MRP2. Indeed, upon the deletion of the *P. berghei mrp* gene, we observed a phenotype of aborted liver stage development comparable to *Pfmrp2* deletion. The absence of blood infections after injecting high numbers of mutant *P. berghei* sporozoites in mice indicates complete abortion of liver stage development in the absence of *P. berghei* MRP2.

The deletion of the single *P. berghei mrp* gene and the combined deletion of the two *P. falciparum mrp* genes did not result in a detectable effect on asexual blood stage multiplication, suggesting that MRP proteins do not play an essential role in asexual stages. In apparent contradiction, we have previously demonstrated that *P. falciparum* MRP1 and MRP2 are located on the plasma membrane of blood stage parasites[17]. However, we were unable to detect *Pb*MRP using transgenic *P. berghei* parasites expressing cmyc-tagged *Pb*MRP. Since we were able to detect *Pbmrp::cmyc* in hepatocytes, failure to detect it in blood stages suggests that *Pb*MRP is either not expressed or that the protein expression level is too low for detection by immunofluorescence. MRP transporters do not have an essential function during blood stage multiplication, or alternatively other subfamily transporters are able to compensate for MRP function, sensitivity of the applied assays is too low or tested conditions deviate from natural infections. A previously reported *P. falciparum* mutant lacking MRP1 showed a reduced growth of blood stages at higher parasite densities[64]. Parasitaemias >5% could not be obtained in cultures of this mutant when medium was replaced once a day, which could be increased to a maximum of 7% in case culture medium was replaced twice a day. In contrast, we readily obtained parasitaemias between 10-20% in cultures of MRP1-deficient parasites using the semi-automated culture system where medium is changed twice daily[206]. These findings indicate that culture conditions may indeed act upon growth kinetics of MRP-deleted parasites. However, in the light of the wide range of MRP substrates in other eukaryotes, it is also possible that MRP2 transports molecules in the liver cell that it does not in the blood. For example, human MRPs are known to transport factors that are involved in intracellular signaling pathways, which may profoundly affect parasite development in the nucleated hepatocyte but not anucleate RBCs.

MRP1 has been associated to sensitivity of *P. falciparum* to anti-malarial drugs[11,57,58]. Single nucleotide polymorphisms (SNP) in MRP1 and upregulation of expression of MRP1 and MRP2 alter drug-sensitivity profiles, possibly by increased substrate affinity and export of antimalarials. Using the chloroquine-resistant W2 strain, Raj *et al.* found an increase in sensitivity of MRP1-deficient mutants to chloroquine, quinine, artemisinin, piperaquine and primaquine, resulting in a decreased IC_{50} concentration compared to parent WT parasites[64]. We did not detect changes in drug-sensitivity profiles of the three *P. falciparum* mutants lacking MRP proteins in the chloroquine-sensitive NF54 background, except for a small decrease in mefloquine sensitivity. The differential use of *Pf* parasite background lines may be responsible for this discrepancy. Moreover, we removed the selectable

marker though FLPe mediated recombination, whereas the deletion of *mrp1* by Raj *et al.* was exerted in a single crossover fashion, and heterologous DNA including the selectable marker was maintained.

Immunofluorescence analysis of liver stages of both *P. falciparum* and *P. berghei* indicate that MRP2 is localized at the plasma lemma membrane of liver stage parasites, a site consistent with ABC transporters of other eukaryotic cells, and it is here that they are usually involved in the extrusion of various compounds. MRP transporters predominantly translocate organic anions. Substrates may include potentially toxic metabolites for which extrusion may be essential for parasite survival or glutathione that plays an important role in intracellular oxidative stress management [87]. Moreover, MRP2 might also be involved in transport of substrates that are involved in host-cell or parasite signaling pathways, which include cyclic nucleotides, leukotrienes and prostaglandins[193-195]. An important role of cGMP signaling has been identified in liver stage development, as merozoite release was inhibited in the absence of cGMP-dependent protein kinase G (PKG), the main mediator of cGMP signaling[241].

As MRP2 is essential for parasite survival during liver stage, this protein may be an attractive drug-target to arrest liver stage development. MRP2 is located at an easily accessible site, and drug resistance mechanisms depending on transporter activity may be evaded[149]. Specific inhibitory compounds that have been identified for human MRP proteins such as MK571 can be used as prototypes for the development of specific and safe *Plasmodium* MRP2 inhibitors[196].

In addition, MRP2-deficient *P. falciparum* parasites may be attractive genetically attenuated parasites (GAP) vaccine candidates. A number of rodent GAPs have previously been reported to arrest at early time points during development in the liver[240,242]. These include GAPs based on genes essential for the formation and maintenance of a parasitophorous vacuole (PV) (*b9*, *p52*, *p36*, *uis3* and *uis4*; [243-245]), type II fatty acid synthesis (i.e. *fabb/f, fabz, pdh e1a*; [246,247]), and regulation of gene expression (*sap1/slarp*[248-250]). Unfortunately, many of the above mentioned target genes show leaky phenotypes, resulting in blood stage infections after administration of high numbers of sporozoites. Incomplete liver stage arrest obviously disqualifies GAPs for further clinical development for safety reasons. The *PbΔmrp2* gene deletion mutant, however, showed no breakthrough to blood stage infection upon injection with a high doses of sporozoites in C57bl/6 mice, the most sensitive mouse

strain. The only alternative GAP candidate that arrests completely during liver stage is lacking the *sap1*/*slarp* gene[249-252]. This GAP arrests early after hepatocyte invasion as these parasites do not construct a functional PV[249,250], however, for rodent GAPs it has been shown that immunization with parasites arresting later during liver stage induces a superior immune response presumably through the prolonged presentation of a larger repertoire of antigenic stimuli[253,254.] Parasites lacking MRP2 develop to a much further extent and persist in hepatocytes as the PV is correctly formed and maintained. The prolonged presentation of a larger repertoire of antigens including PV-associated proteins renders mutant parasites lacking MRP2 attractive GAP candidates.

In conclusion, whereas MRP proteins are redundant for blood stage and sporogonic development, parasites lacking MRP2 fail to produce mature liver stage parasites. The MRP2 protein is therefore an interesting target for development of drugs targeting *Plasmodium* liver stages. Furthermore, safety and immunogenicity of parasites lacking the *mrp2* gene should be further investigated to explore the possibilities of applying these parasites as GAP candidates in a whole parasite vaccination strategy.

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B: probes and other PCRs

Gene	Construct	Basic construct	Primer	Sequences	Restriction sites	Description
Pfmrp1			SR009	GACGACATATAAAGA AAATGTTGG		mrp1 F iPCR
			SR010	CTTGCATCATTTGAA TTACAATGG		mrp1 R iPCR
			SR011	CAAATGCATGAATATTGG		mrp1 F LR PCR
			SR012	TCAAAATAATCAACTAGCC		mrp1 R LR PCR
Pfmrp2			SR013	CAGAATGATCAGAA TGAGCAAAGCG		mrp2 F iPCR
			SR014	GCCTGTACACGTTA AACATGTCG		mrp2 R iPCR
			SR015	CATGACAAGAATAA TAACGAC		mrp2 F LR PCR
			SR016	CTCAATAAGGAG GATTCATC		mrp2 R LR PCR
Pbmrp			136	GGTATTATCCTTGA ATTTATTTATG		mrp2 orf F, RT-PCR
			138	GGTCGCCACTATTA TTTCG		mrp2 orf R, RT-PCR
			644	GAACAAATTACTT CATTCATAGC		a/b-large subunit (lsu) rrna, probe
			692	CTTATATATTTATAC CAATTG		3'UTR dhfr-ts, F probe
			693	GTTTTTTTTTAATTT TTCAAC		3'UTR dhfr-ts, R probe
			5092	GGGAATTATATAGA TAAGAATAATGAGGG		5' UTR mrp2, F probe
			5093	GCTTGTTTGAAAGTA TAAGTCAATGCCGC		5' UTR mrp2, R probe
			5094	GCGGTCTGATAATGA AATTATTGATGCT		3'mrp2 orf, F probe
			5095	GGTGCCTGGTCCT GTATAATATTAGC		3'mrp2 orf, R probe

Table S1: Details of DNA constructs and primers used in this study
F: Forward
R: Reverse
iPCR: internal PCR
ORF: Open Reading Frame
LR: Long Range

P. falciparum in vitro	WT	*PfΔmrp1*	*PfΔmrp2*	*PfΔmrp1Δmrp2*
Chloroquine	12.9 (10.1 - 16.4)	10.7 (9.14 - 12.5)	9.25 (8.50 - 10.1)	13.6 (9.21 - 20.1)
Artemisinin	39.8 (26.3 - 60.3)	22.0 (17.5 - 27.7)	19.6 (17.3 - 22.1)	39.1 (23.3 - 65.6)
Dihydroartemisinin	42.9 (21.8 - 84.2)	30.9 (20.1 - 47.6)	36.2 (26.3 - 49.7)	134 (70.3 - 256)
Atovaquone	3.69 (2.23 - 6.11)	4.19 (3.06 - 5.74)	3.92 (3.32 - 4.63)	4.55 (3.55 - 5.84)
Lumefantrine	60.0 (35.0 - 103)	43.9 (37.6 - 51.3)	69.1 (52.7 - 90.5)	102 (69.1 - 151)
Quinine	25.4 (20.9 - 30.9)	36.2 (26.0- 50.4)	26.5 (20.5 - 34.2)	39.1 (26.9 - 56.9)
Mefloquine	121 (99.2 - 147)	241 (184 - 316)*	242 (148 - 390)*	272 (193 - 382)*

Table S2: Drug sensitivity (IC_{50} values and 95% CI) of *P. falciparum* mutants lacking expression of MRP proteins

[1] Drug sensitivity determined in culture. IC_{50} values are calculated from the dose response curves shown in Figure S3

* Significant different from WT ($p<0.05$)

Parasite count per well (mean and S.D.)

Day p.i.	WT	*PfΔmrp1*	*PfΔmrp2*	*PfΔmrp1Δmrp2*
2	106.5 (61.9)	448.3 (29.4)	347.7 (27.8)	343.7 (141.4)
3	118.5 (68.4)	375.0 (39.6)	64.3 (1.16)	443.0 (18.0)
4	71.8 (56.7)	ND	35.3 (4.73)	37.7 (15.0)
5	60.5 (37.9)	173.7 (9.24)	24.7 (11.0)	11.5 (7.40)
6	44.7 (37.2)	166.3 (22.5)	17.0 (4.36)	2.00 (1.79)
7	28.7 (13.4)	77.0 (4.24)	3.67 (0.58)	0.83 (0.98)
10				0.33 (0.58)

Parasite diameter - µm (mean and S.D.)

Day p.i.	WT	*PfΔmrp1*	*PfΔmrp2*	*PfΔmrp1Δmrp2*
2	2.0 (0.2)	1.8 (0.1)	1.1 (0.1)	2.4 (0.2)
3	3.0 (0.4)	3.0 (0.6)	1.1 (0.2)	ND
4	5.5 (0.8)	ND	1.5 (0.1)	2.2 (0.4)
5	6.3 (0.6)	5.4 (0.6)	1.8 (0.2)	2.3 (0.5)
6	8.4 (1.1)	6.6 (0.2)	2.0 (0.1)	2.3 (0.5)
7	10.6 (2.2)	9.2 (1.3)	2.1 (0.4)	2.4 (0.2)

Table S3: Number and size of *P. falciparum* WT and mutant liver stages in cultures of primary human hepatocytes at different days after infection

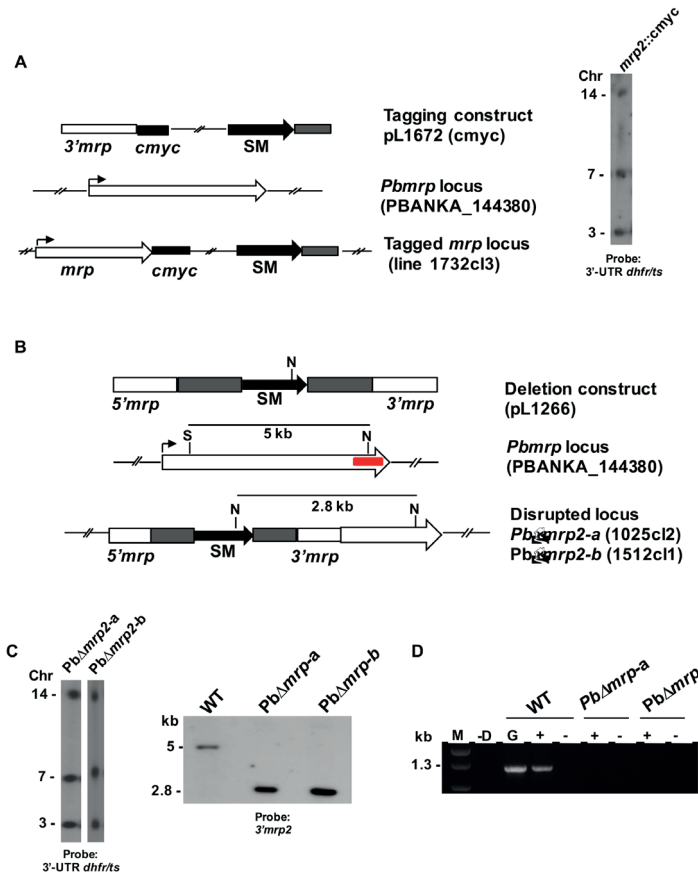


Figure S1. Generation and genotype analysis of *P. berghei Pbmrp::cmyc* and *PbΔmrp2* parasites

A Schematic representation of the DNA construct (pL1672) used to C-terminal tag the *Pbmrp2* gene with cmyc by single cross-over homologous recombination (SM: selectable marker cassette). See Table S3 for primer sequences used to amplify the target region. Southern analysis of separated chromosomes of *Pbmrp::cmyc* (1732cl3) confirms correct integration of the tagging construct. Separated chromosomes were hybridized using an 3'UTR pbdhfr probe that recognizes the DNA-construct integrated into the *mrp2* locus on chromosome 14, the endogenous *dhfr/ts* on chromosome 7 and the GFP-luciferase reporter cassette in the *230p* locus on chromosome 3. **B** Schematic representation of the DNA construct (pL1266) used to disrupt the *Pbmrp2* gene. The construct is aimed at disruption of the target gene by double cross-over homologous recombination at the 5'- and 3'UTR regions (SM: selectable marker cassette). Two gene-deletion mutants were selected from two independent transfections, *PbΔmrp2-a* (line 1025cl2) and *PbΔmrp2*-b (line 1512cl1). See Table S3 for primer sequences used to amplify the target regions. The DNA-probe (red) and restriction sites used for Southern analysis (see C) are indicated: *Nhe I* (N) and Sca I (S). **C** Southern blot analysis of separated chromosomes and digested genomic DNA confirm correct deletion of the *mrp2* gene (left). Separated chromosomes were hybridized using an 3'UTR pbdhfr probe that recognizes the DNA-construct integrated into the *mrp2* locus on chromosome 14, the endogenous *dhfr/ts* on chromosome 7 and the GFP reporter cassette (*PbΔmrp2*-a; 1025cl2) or the GFP male- RFP female reporter cassette (*PbΔmrp2*-b; 1512cl1) in the *230p* locus on chromosome 3. *Nhe I* (N) and *Sca I* (S) digested DNA was hybridized (right) with a 3' *mrp2* probe (see B for the location), recognizing the expected DNA fragments indicated in C (a 5 kb fragment in WT and a 2.8 kb fragment in the PbΔmrp2 lines). **D** RT-PCR analysis of *mrp* transcription in WT and *PbΔmrp2* parasites using *mrp2* specific primers (Table S3). As a control for gDNA contamination, PCRs were carried out on cDNAs synthesized in the presence (+) or absence (-) of reverse transcriptase. WT gDNA and cDNA show amplicons of the expected size whereas no products were amplified in the mutant parasites. No cDNA (-D) and WT gDNA (G) were used as negative and positive controls, respectively.

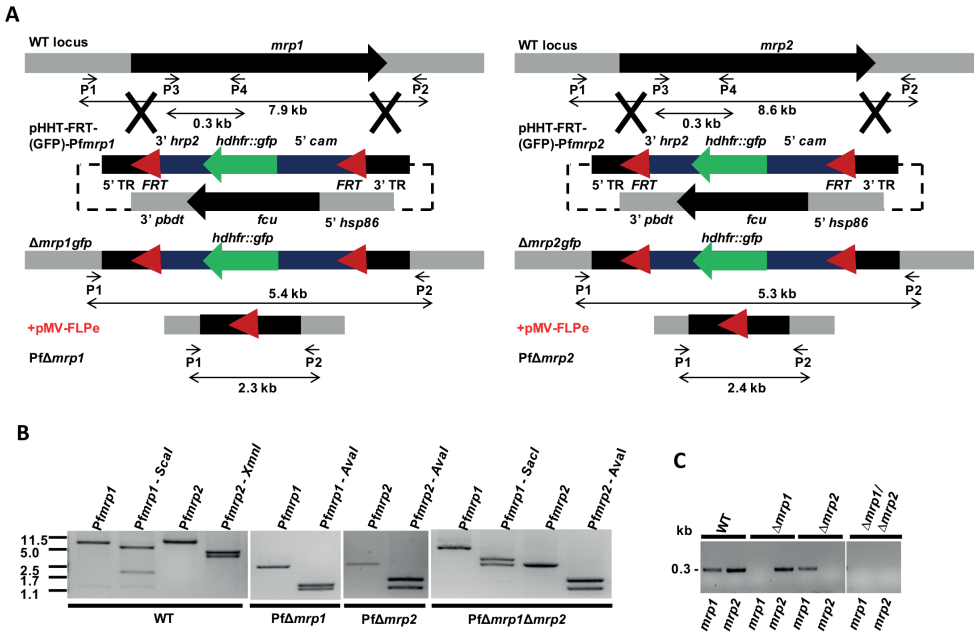


Figure S2. Generation and genotype analysis of *P. falciparum* PfΔmrp1, PfΔmrp2, and PfΔmrp1Δmrp2 parasites.

A Schematic representation of the genomic loci of *mrp1* (PF3D7_0112200), and *mrp2* (PF3D7_1229100) genes of wild-type (WT), *PfΔmrp1* and *PfΔmrp2* gene deletion mutants before (*Δmrp1gfp* and *PfΔmrp2gfp*) and after the FLPe mediated removal of the *hdhfr::gfp* resistance marker (*PfΔmrp1* and *PfΔmrp2* clones) respectively. The constructs for the targeted deletion of *mrp* genes (pHHT-FRTGFP *mrp*) contain two FRT sequences (red triangles) that are recognized by FLPe. P1, P2 and P3, P4 primer pairs for (LR-)PCR analysis of *mrp* loci respectively (see B, C); cam: calmodulin; hrp: histidine rich protein; hsp: heatshock protein; fcu: cytosine deaminase/uracil phosphoribosyl-transferase; hdhfr::gfp: human dihydrofolate reductase fusion with green fluorescent protein; pbdt: P.berghei dhfr terminator.

B Long range PCR analysis of genomic DNA from WT, *PfΔmrp1*, *PfΔmrp2* and *PfΔmrp1Δmrp2* parasites confirms the *mrp* gene deletions in the different mutants and subsequent removal of the *hdhfr::gfp* resistance marker. The PCR products are generated using primers P1 and P2 (see a for their location and Table S3 for the sequences). A PCR product for WT *mrp1* and *mrp2* of 7937 and 8595 bp was obtained, whereas amplification of *PfΔmrp1* and PfΔmrp2 genomic DNA resulted in product sizes of 2257 and 2380 bp. For the *PfΔmrp1Δmrp2* double gene deletion mutant, a PCR product of 5353 bp was obtained upon *Pfmrp1* amplification, as the selectable marker at this site was not removed. Specific products were obtained upon restriction with *ScaI* and *XmnI* for wild type *mrp1* and *mrp2* PCR products respectively; *AvaI* restriction of both amplicons obtained from the *PfΔmrp1* and *PfΔmrp2* single gene deletion mutants and *SacI* and *AvaI* restriction of the PCR product obtained after *mrp1* and *mrp2* amplification in the *PfΔmrp1Δmrp2* line. C PCR analysis of genomic DNA from WT, *PfΔmrp1*, *PfΔmrp2* and *PfΔmrp1Δmrp2* parasites confirms the *mrp* gene deletions in the different mutants. The PCR products are generated using primers P3 and P4 (see A for their location and **Table S3** for the sequences).

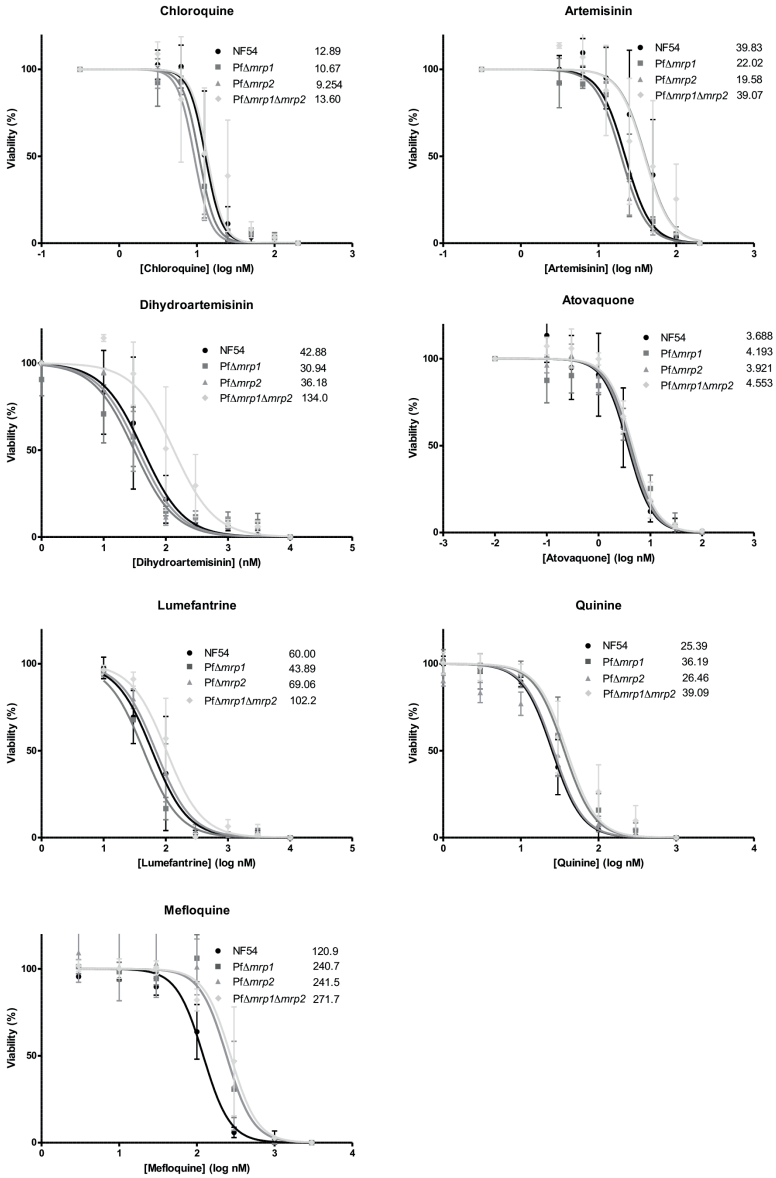


Figure S3. Sensitivity of WT and mutant parasites to antimalarial drugs
Dose-response curves of blood stages of WT, *PfΔmrp1*, PfΔmrp2 and *PfΔmrp1Δmrp2* cultured in the presence of the following antimalarials: chloroquine, artemisinin, dihydroartemisinin, atovaquone, lumefantrine, quinine and mefloquine. No significant differences in IC_{50} concentrations were observed when the IC50 values of the different mutants were compared with those of WT, except for mefloquine (see **Table S1**).

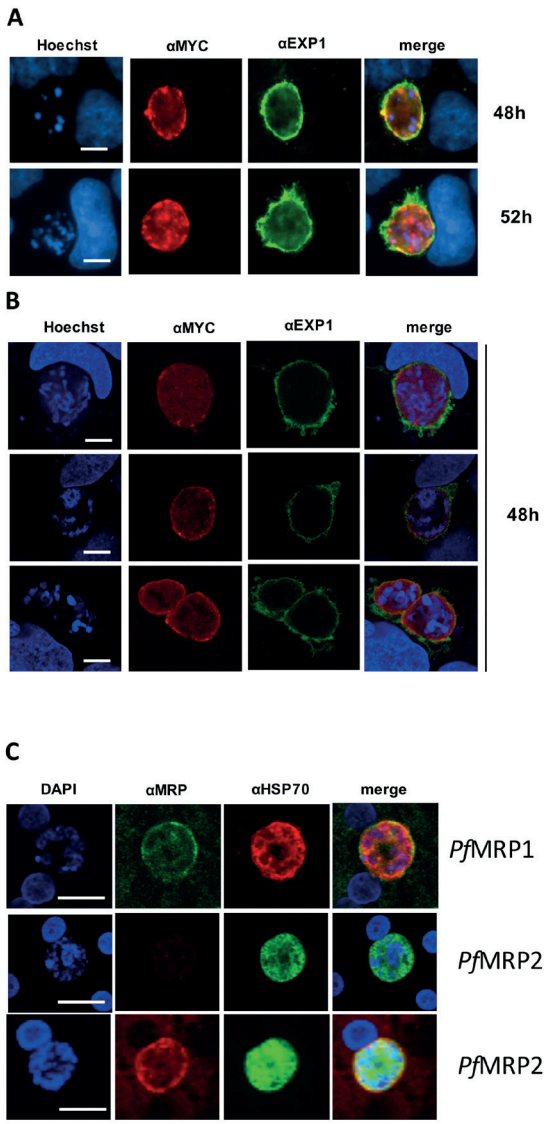
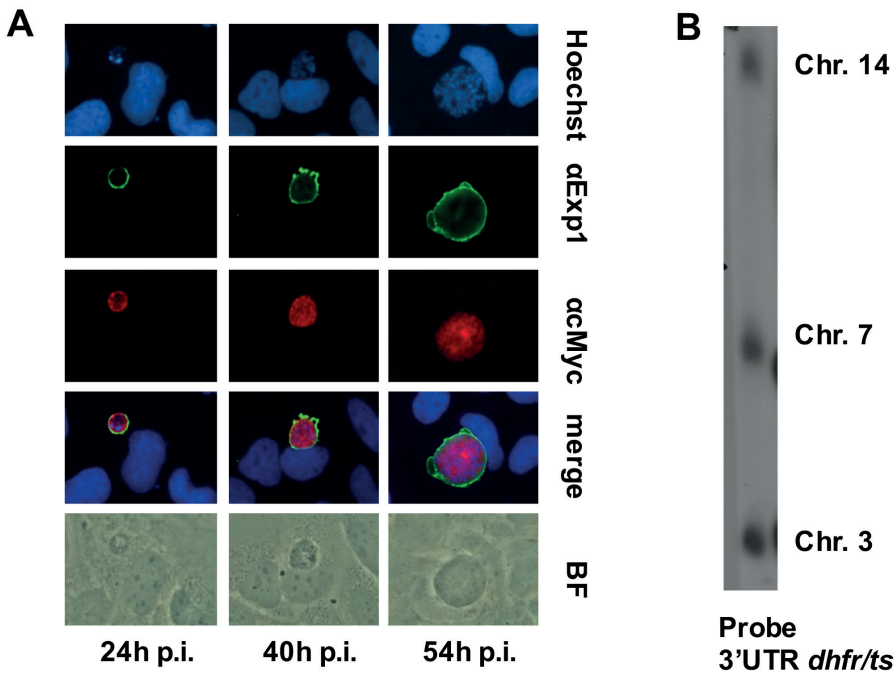
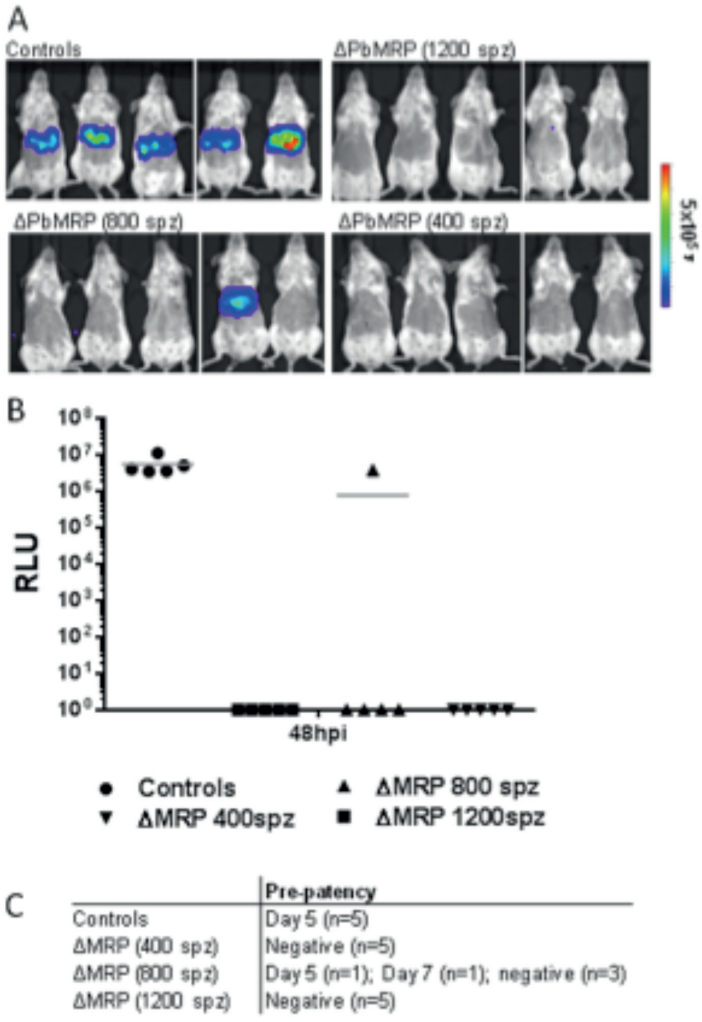


Figure S4. Localisation of MRP2 in liver stages of *P. berghei Pbmrp::cmyc* by fluorescence microscopy, and localisation of MRP1 and MRP2 in liver stages of *P. falciparum* NF54 WT parasites
Standard **(A)** and confocal **(B)** fluorescence microscopy images of *Pbmrp::cmyc* liver stages at different time points (hours; h) after invading cultured hepatocytes. *Pbmrp::cmyc* liver stages were stained with antibodies recognizing cmyc (red) and the parasite vacuole membrane protein EXP1 (green). Circumferential staining with the cmyc antibodies which does not co-localise with EXP1 staining indicates a location of MRP2::cmyc at the plasma lemma membrane of the parasite. Nuclei are stained with Hoechst-33342. Images were acquired with confocal microscope Leica TCS SP8 X with white light laser (WLL) and DM RA Leica fluorescence microscope. Scale bar 10µm. **C** Confocal microscopy was used for localization of MRP1 and MRP2 in NF54 WT liver stage parasites at day 6 using antibodies recognizing MRP1 (green or red) and MRP2 (red) and cytosolic HSP70 shows a honeycomb-like pattern for MRP proteins, indicating localization of these proteins on the plasma lemma membrane of the parasite.



Line	Wild type (n=6)	cmyc::MRP2 (n=6)
Prepatent period	5	3

Figure S5. Liver stage development of *Pbcmyc::mrp2*.
A Immunofluorescence analysis of liver stages at different time points post infection (p.i.) of cultured hepatocytes (upper panel). Parasites are stained with anti-cmyc and anti Exp1 antibodies and nuclei with Hoechst. At 40 and 54 hour dividing liver stages are detected inside the PVM. Infection of mice by intravenous injection of 104 *Pbcmyc::mrp2* sporozoites results in a prepatent period similar to mice infected with wild type parasites (lower panel). **B** Genotyping by Southern analysis of PFG-separated chromosomes (chr) of *Pbcmyc::mrp2* blood stage parasites after mosquito infection shows the same genotype as before mosquito transmission (see Figure S1). The probe (3'UTR *dhfr/ts*) hybridizes to the endogenous *dhfr/ts* gene on chromosome 7, the GFP-Luciferase expression cassette in chromosome 3 and the *Pbcmyc::mrp2* tagging construct in chromosome 14.



C

	Pre-patency
Controls	Day 5 (n=5)
ΔMRP (400 spz)	Negative (n=5)
ΔMRP (800 spz)	Day 5 (n=1); Day 7 (n=1); negative (n=3)
ΔMRP (1200 spz)	Negative (n=5)

Figure S6. Protection against liver stage parasitemia after vaccination with low-dose gene deletion parasites

BALB/c mice were vaccinated with saline, 400, 800 or 1200 gene-deletion sporozoites, and after 21 days they were challenged with luminescence-expressing sporozoites. **A** Development of liver stage parasitemia after 48 hours is displayed. **B** Luminescence intensity was measured for each mouse. **C** Appearance of blood stage parasitaemia was monitored.

CHAPTER 5

Vital and dispensable roles of *Plasmodium* multidrug resistance transporters during blood- and mosquito-stage development

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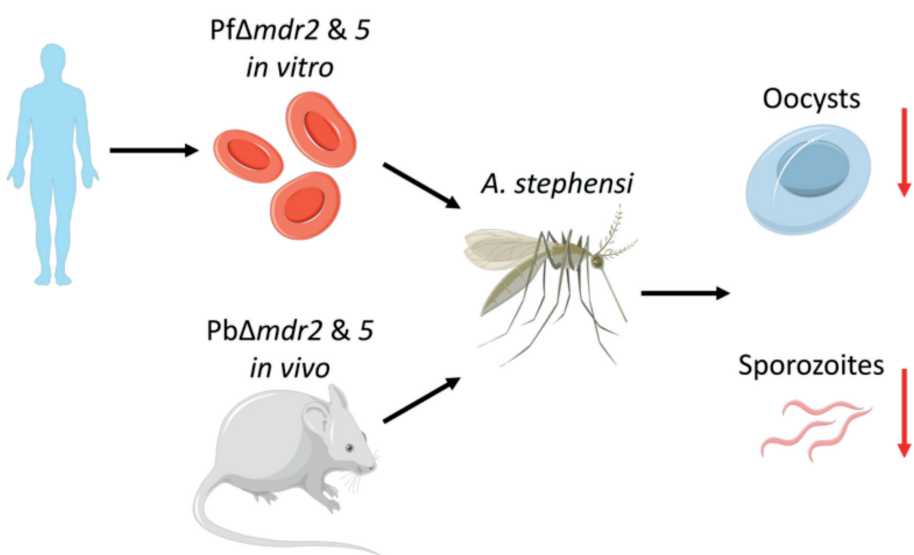
ABSTRACT

Multidrug resistance (MDR) proteins belong to the B subfamily of the ATP Binding Cassette (ABC) transporters, which export a wide range of compounds including pharmaceuticals. In this study we used reverse genetics to study the role of all seven *Plasmodium* MDR proteins during the life cycle of malaria parasites. Four *P. berghei* genes (encoding MDR1, 4, 6 and 7) were refractory to deletion, indicating a vital role during blood stage multiplication and validating them as potential targets for antimalarial drugs. Mutants lacking expression of MDR2, MDR3 and MDR5 were generated in both *P. berghei* and *P. falciparum*, indicating a dispensable role for blood stage development. Whereas *P. berghei* mutants lacking MDR3 and MDR5 had a reduced blood stage multiplication *in vivo*, blood stage growth of *P. falciparum* mutants *in vitro* was not significantly different. Oocyst maturation and sporozoite formation in *Plasmodium* mutants lacking MDR2 or MDR5 was reduced. Sporozoites of these *P. berghei* mutants were capable of infecting mice and life cycle completion, indicating the absence of vital roles during liver stage development. Our results demonstrate vital and dispensable roles of MDR proteins during blood stages and an important function in sporogony for MDR2 and MDR5 in both *Plasmodium* species.



INTRODUCTION

ATP Binding Cassette (ABC) transporters are membrane proteins that translocate diverse compounds at the expense of ATP and have been well conserved in many organisms. They are organized into either two domains (half-transporter), consisting of a transmembrane domain (TMD) and a nucleotide-binding domain (NBD) required for ATP-hydrolysis or into four domains (full-transporter), consisting of two TMDs alternated with two NBDs **(Figure 1A)**. Half-transporters require homo- or hetero-dimerization to become functional[20].

In humans, ABC-transport proteins are well known for their involvement in multidrug resistance against antiviral, antimicrobial or anti-cancer drugs[255]. Especially transporters of the ABCB, ABCC and ABCG subfamilies play important roles in resistance, of which the most studied multidrug resistance (MDR) protein is MDR1. This protein belongs to the ABCB subfamily, which contains both half- and full-transporters[255]. Amplification of the MDR1 encoding gene has been shown to confer resistance of cancer cells to chemotherapeutic drugs through increased excretion[256]. However, these transporters also play important roles in physiological cellular processes such as membrane biogenesis, autocrine pathways and homeostasis, as their substrates may vary from lipids and sterols to heavy metals and amphipathic or conjugated xenobiotic compound [87].

In the human malaria parasite *P. falciparum*, 16 ABC genes have been identified[148]. Based on phylogenetic analysis of the conserved nucleotide binding domains, seven are recognized as members of the B family of ABC transporters. *Pfmdr1* (PF3D7_0523000) has been most intensively studied for its involvement in antimalarial drug resistance. Amplifications and polymorphisms of *Pfmdr1* have been associated with decreased sensitivity towards multiple antimalarial drugs, including chloroquine and artemisinin[12,24,59]. Its primary localization on the membrane of the digestive vacuole may indicate involvement in drug accumulation, where several antimalarial compounds interfere with polymerization of reactive free heme released upon hemoglobin digestion[257]. Hence, *Pfmdr1* polymorphisms have been shown to reduce drug accumulation resulting in antimalarial resistance[12]. Furthermore, a single nucleotide polymorphism (SNP) in *Pfmdr2* (PF3D7_1447900), a heavy metal transporter[48], is associated with *in vitro* resistance to pyrimethamine [50]. Recently, another SNP in *Pfmdr2* showed a statistically significant association ($p = 2 \times 10\text{-}10$) with artemisinin resistance[258]. Moreover, varying indels in the *Pfmdr6* gene (PF3D7_1352100) have been associated with altered artesunate and piperaquine

sensitivity[11,51-53], although transfection-based confirmation of that association is lacking. Apart from a role in drug resistance, the physiological function as well as substrate specificity of the *Plasmodium* MDR proteins remains largely unknown, also for *Pf*MDR5 (PF3D7_1339900), which is localized at the plasma membrane of intra-erythrocytic parasites[17].

To gain insight into the function of these proteins and their role in the *Plasmodium* life cycle, we first systematically targeted the seven MDR proteins by experimental genetics in the murine malaria model parasite *Plasmodium* berghei. We found that four out of seven ABCB encoding genes (*mdr1*, *mdr4*, *mdr6* and *mdr7*) are refractory to gene deletion. We were able to select *P. berghei* mutants lacking *mdr2*, *mdr3* and *mdr5*, which is indicative of a dispensable role for the encoded proteins during asexual blood stage growth *in vivo*. We subsequently targeted *mdr2*, *mdr3* and *mdr5* for deletion in *P. falciparum* and were able to select mutants *in vitro*, confirming a non-essential role for blood stage development. Both *P. berghei* and *P. falciparum* mutants lacking expression of MDR2 and MDR5 showed reduced oocyst formation. Additionally, the mutants lacking *Pbmdr2*, *Pbmdr5* and *Pfmdr2* showed reduced sporozoite production. Furthermore, *Pb*MDR2::mCherry expression was observed during blood- and mosquito stage development. Combined, our results provide evidence that *Plasmodium* express several MDR proteins that have specific functions during sporogony.

MATERIALS AND METHODS

Experimental animals and *P. berghei* ANKA reference lines

Animal experiments were conducted in female C57BL/6, BALB/c and Swiss OF1 mice (6-8 weeks; Charles River) and approved by the Animal Experiments Committee of the Leiden University Medical Center (DEC; 12042; 12111), or in C57BL/6 mice as approved by the ethics committee of the Berlin state authority (Landesamt für Gesundheit und Soziales Berlin, permit number G0469/09. Both the Dutch Experiments on Animal Act and the German 'Tierschutzgesetz in der Fassung vom 22. Juli 2009' are established under European guidelines (EU directive no. 86/609/EEC regarding the Protection of Animals used for Experimental and Other Scientific Purposes).

Two different *P. berghei* ANKA parasite lines were used to generate gene-deletion mutants: 676m1cl1 (PbGFP-LUCcon; mutant RMgm-29; www.pberghei.eu) and 1037cl1 (PbGFP-Lucschiz; mutant RMgm-32; www.pberghei.eu), originating from the cl15cy1 wild type (WT)[204] which was

used to generate the *Pbmdr2*::mCherry tagged parasites. In both reporter lines, a *gfp-luc* expression cassette is stably integrated into the *Pb230p* locus in absence of a drug-selectable marker[207,214]. These lines thus express the GFP-Luciferase fusion protein under the control of either the constitutively active *eef1a* promoter (676m1cl1) or the schizont-specific *ama1* promoter (1037cl1).

Generation of *P. berghei* ABC transporter gene deletion and tagged mutants

Genes encoding ABC transporters were deleted using a double cross-over strategy based on homologous recombination of targeting constructs into the genome of the parasite. Targeting sequences were PCR amplified from *P. berghei* ANKA (cl15cy1) genomic DNA using primers specific for the 5'UTR or 3'UTR regions of the different ABC transporter genes **(Table S3).** These targeting regions (TR) were cloned into the pL0001 plasmid (www.mr4.com), flanking the pyrimethamine resistant T*oxoplasma gondii* (*Tg*) dihydrofolate reductase-thymidylate synthase (*dhfr/ts*) as a selectable marker (SM) under control of the *P. berghei dhfr/ts* promoter. Tagging of *mdr4* was performed using a similar double crossover recombination strategy as described above. A DNA construct containing an 3xHA tag was used to target *mdr4*. This construct was obtained from the Sanger Institute (PbGEM-084058; http://plasmogem.sanger.ac.uk/designs/final_vector/84058; named in this study plasmid pL1995)[259]. This construct aims at integration at the 3' end of *mdr4*, replacing the stop codon with an HA tag and contains the pyrimethamine resistant human dihydrofolate reductase-thymidylate synthase (*hdhfr*). Transfection and pyrimethamine selection of mutant parasites with was performed as described for generation of the gene deletion mutants.

For the *Pbmdr2*::mCherry tagging construct, fragments at the 3' region and the carboxy-terminal end of the coding sequence were amplified using specific primers **(Table S3)**. These fragments were cloned into the pBAT-SIL6 vector2[60] using the indicated restriction enzymes **(Table S3)**. The carboxy-terminus was cloned in frame with the *mCherry-3xMyc* tag. Gene-deletion and tagging constructs were verified by Sanger sequencing and linearized with the appropriate restriction enzymes **(Table S3)**. Transfection and selection of transformed parasites with pyrimethamine was performed using the high efficiency transfection and selection technology for genetic modification of *P. berghei* [204]. Clonal lines of all gene-deletion mutants were generated through limiting dilution of the parasites in mice. Deletion of the targeted genes or *mdr2* tagging by correct integration of the DNA constructs was verified by diagnostic PCR analysis covering the 5'TR and 3'TR integration using gene-specific primers **(Table S4)** and Southern analysis of pulsed-field gel electrophoresis separated chromosomes that were hybridized with a 3'pb*dhfr/ts* probe (FIGE) **(Table S4)**.

Transcription of the *mdr* genes was determined by Northern analysis of RNA obtained from WT and gene-deletion mutant blood stages of asynchronous *in vivo* infections or from synchronous blood infections of WT parasites [208]. Northern blots were hybridized with a 5'-UTR fragment of the *mdr* genes, PCR amplified from genomic WT DNA using the appropriate primer pairs **(Table S3)** and with the *a/b-large subunit rRNA* probe as control (primer 644) [11].

***In vivo* multiplication rate of asexual *P. berghei* blood stage parasites**

During the cloning procedure of the gene-deletion mutants, the multiplication rate of asexual blood stage parasites in mice was determined as described before[214]. Parasitemias in percentages in Swiss OF1 mice injected with a single parasite are determined at day 8 to 11 in Giemsa-stained blood films. Per mouse, an estimated number of 1.2 x 10^{10} erythrocytes is used to calculate the 24-hour multiplication rate. The percentage of infected erythrocytes in mice infected with reference lines of the *P. berghei* ANKA strain consistently ranges between 0.5-2% at day 8 post infection, resulting in a mean multiplication rate of 10 per 24h[261].

***In vivo P. berghei* gametocyte production and *in vitro* ookinete production**

The gametocyte conversion rate was determined as the percentage of ring forms that develop into mature gametocytes in synchronized infections in mice that are pre-treated with phenylhydrazine-HCl[208]. Ookinete production was analyzed by standard *in vitro* fertilization and ookinete maturation assays[262]. Gametocytes for these assays were obtained from infected mice that had been pre-treated with phenylhydrazine-HCl to increase gametocyte numbers. The ookinete conversion rate is defined by the percentage of female gametes that develop into mature ookinetes under standardized *in vitro* culture conditions for activation of gametocytes, fertilization and ookinete maturation. The percentage of females that developed into ookinetes was determined by counting female gametes and mature ookinetes in Giemsa-stained blood smears, made at 16-18 h post activation.

***P. berghei* oocyst and sporozoite production in *Anopheles stephensi* mosquitoes**

For mosquito transmission experiments, female *A. stephensi* mosquitoes were fed on mice infected with WT parasites or gene-deletion mutants. Oocyst development, oocyst production and sporozoite production was monitored in infected mosquitoes as described[263]. Oocyst numbers were counted and measured in midguts of infected mosquitoes at 9, 12, 14, 16, 19, 21, and 23 days post infection, while sporozoite numbers

were counted at day 21-22. Salivary gland sporozoites were isolated and counted as described[215]. Mean differential oocyst (± SEM) and sporozoite numbers between WT and mutant lines were determined using a one-way ANOVA with Dunnett's post-test.

Localization of HA-tagged *Pb*MDR4 and mCherry-tagged *Pb*MDR2

Detection of the HA-MDR4 protein was performed after fixing asexual parasites with 4% para-formaldehyde (PFA), quenching with 0.1 M glycine, blocking with 10% FCS and permeabilization with 1% Triton-X-100. Localization of was performed using specific antibodies against HA raised in rat (Roche, 1:1000), visualized with anti-rat ALEXA488 (green). Nuclei were stained with DAPI (red).

Live protein localization of the *Pb*MDR2::mCherry tagged protein was performed only minutes after sample collection and Hoechst 33342 DNA staining, using either conventional slides and coverslips or concanavalin A-coated ibidi µ-Dishes (35 mm, low; Grid500) with pre-warmed RPMI 1640 medium containing 20% fetal calf serum. Images for live protein localization were recorded on a Zeiss AxioObserver Z1 epifluorescence microscope, equipped with a Zeiss AxioCam MRm camera, and processed minimally with FIJI[264].

***P. berghei* sporozoite infectivity and liver stage development**

P. berghei sporozoites were collected at day 21-22 post infection by hand-dissection of the salivary glands. Salivary glands were collected in DMEM (Dulbecco's Modified Eagle Medium from GIBCO) and homogenized in a home-made glass grinder. The number of sporozoites was determined by counting these in 10 salivary glands in duplicate in a Bürker-Türk counting chamber using phase-contrast microscopy.

To determine *in vivo* infectivity of sporozoites, Swiss OF1 mice were infected with 104 salivary gland sporozoites by intravenous injection, as previously described[263]. Blood stage infections were monitored by analysis of Giemsa-stained thin smears of tail blood collected on day 4–8 after inoculation of sporozoites. The prepatent phase (measured in days post sporozoite infection) ends at the day that blood stage infection with 0.5-2% parasitemia is observed.

P. falciparum reference lines and culture conditions

Plasmodium falciparum wild type (NF54) and mutant parasites were maintained in a semi-automated culture system and gametocyte formation was induced as reported previously[153,205,210]. Briefly, in vitro parasites were grown in RPMI medium supplemented with human serum (complete medium) and 5% hematocrit. Medium was changed twice daily and fresh human red blood cells were obtained weekly from the Dutch national blood bank (Sanquin).

Generation and genotyping of *P. falciparum mdr2*, *mdr3* and *mdr5* gene deletion mutants

For phenotype analysis, we used the previously generated *P. falciparum mdr2* and *mdr5* gene deletion lines lacking the *hdhfr::gfp* selectable marker[265]. In addition, for the parasite development within mosquitoes, we used two independetly generated additional *PfΔmdr2* and *PfΔmdr5* clones in which the selectable marker was not removed. For deletion of the *P. falciparum mdr3* and *mdr6* genes, we followed a homologous double crossover strategy as described[151,266]. The deletion construct was made by replacing the *Pf52* homologous regions in the pHHT-FRT-(GFP)-*Pf52* construct (kindly provided by Ben van Schaijk) with *mdr3* and *mdr6* target regions (TR) **(Figure S1)**[152]. These regions were amplified from *P. falciparum* NF54 genomic DNA (gDNA) using PfuUltra II Fusion HS DNA Polymerase (Agilent Technologies) with primer pairs for the 5′ and 3′ target regions of *mdr3*, respectively **(Table S3)**, and a similar approach was applied to amplify the *mdr6* target regions. The 5′ and 3′ target regions were cloned into the pHHT-FRT-(GFP)-Pf52 construct using *BssHII* plus *BsiWI* and *XmaI* plus *NheI* restriction enzymes after TOPO TA subcloning (Invitrogen) and sequence validation. This resulted in the deletion constructs pHHT-FRT-(GFP)-*Pfmdr3* and pHHT-FRT-(GFP)-*Pfmdr6*. Transfection and selection procedures we performed as described previously[151,266], and clonal lines were obtained by limiting dilution.

Genotyping of the *PfΔmdr3* mutant was performed by diagnostic PCR using Expand Long Range dNTPack (Roche) PCR (LR-PCR). Mixed asexual blood stage gDNA from NF54 wild type (WT) and *PfΔmdr3* was isolated using the QIAamp DNA Blood Mini Kit (Qiagen). Primers flanking the 5′ and 3′ TR of *mdr3* were used to amplify WT and mutant DNA using LR-PCR to validate correct double homologous crossover integration **(Table S4).** The LR-PCR was performed as described previously[265], with an annealing temperature of 43.5°C. An additional diagnostic PCR was performed as reported[265] on gDNA using primers designed within the open reading frame (ORF) of *mdr3* **(Table S4).**

***P. falciparum in vitro* multiplication rate of asexual blood stages**

Asexual growth of *P. falciparum* WT and two clones from each of the mutant lines *PfΔmdr2*, *PfΔmdr3* and *PfΔmdr5* was monitored in three consecutive experiments during 7 days. Briefly, parasites were inoculated in triplicate at 0.1% parasitemia in complete medium (at 2.5% hematocrit) and were incubated in 96-wells plates at 37°C under candle jar culture conditions[267]. After sedimentation of RBC, complete medium was refreshed daily. Each day, a subset of iRBC samples was resuspended and transferred to black-side clear-bottom 96-wells cell culture plates (Greiner Bio-One) and frozen at -20°C until readout using a modified *Plasmodium* lactate dehydrogenase (pLDH) method[154]. In this assay, pLDH activity was determined by adding 70 µL fresh reaction mixture (286 mM 3-acetyl pyridine adenine dinucleotide (Sigma-Aldrich), 5.66 U/mL diaphorase (Worthington), 357.5 µM resazurin (Sigma-Aldrich), 286 mM sodium L-lactate (Sigma-Aldrich), 20 mM Tris-HCl pH 8.0) to all plates of three independent experiments. After incubation in the dark for 30-60 min following excitation at 530 nm, absorbance was measured at 590 nm using a Synergy 2 Multi-Mode Microplate Reader (Bio-Tek). Uninfected red blood cells served as background and growth rate was measured in relative fluorescent units (RFU) and plotted using GraphPad Prism version 5.03 (GraphPad Software). Difference in growth rate of all mutant lines (two independently transfected clones per mutant line were pooled) was compared to WT values at all time points using 2-way ANOVA with Bonferroni's post-test.

***P. falciparum* oocyst and sporozoite production in *Anopheles stephensi* mosquitoes**

To study parasite mosquito development of the *PfΔmdr2*, *PfΔmdr3* and *PfΔmdr5* mutant lines, standard membrane feeding assays using female *A. stephensi* mosquitoes were performed as described[153,219]. Midgut oocysts were counted at day 7 post infection in wild type NF54, *PfΔmdr2*, *PfΔmdr3* and *PfΔmdr5* parasites from 7, 6, 1, and 4 independent experiments containing a total of 120, 120, 20 and 60 mosquitoes, respectively. Salivary gland sporozoites were determined at day 14-16 or 18 post infection in 10 mosquitoes per experiment (of which the means are represented in the graph) in 6, 3, 3, and 3 independent assays for NF54, *PfΔmdr2*, *PfΔmdr3* and *PfΔmdr5*, respectively. For *PfΔmdr2* and *PfΔmdr5*, two independent clones were used and the resulting oocyst and sporozoite data was plotted using GraphPad Prism version 5.03 (GraphPad Software). We only included experiments in which at least 70% of the mosquitoes were infected. Mean differential oocyst and sporozoite numbers (± SEM) between WT and mutant lines were determined using a one-way ANOVA with Dunnett's post-test.

RESULTS

Dispensable roles of *P. berghei* MDR2, MDR3 and MDR5 for blood stage development

We systematically targeted all seven mdr genes by experimental genetics in the rodent malaria parasite *Plasmodium* berghei. Multiple attempts to disrupt *Pbmdr1*, *4, 6*, and *7* by DNA constructs that integrate through homologous double cross-over integration **(Figure 1A)** using standard methods for genetic modification of *P. berghei* [204] were unsuccessful **(Table S1)**. Mutant parasites lacking these MDR encoding genes could not be isolated, indicating an essential role of these proteins for blood stage multiplication. Evidence for expression of these genes in blood stages has previously been reported **(Table S2)**. We successfully targeted the *Pbmdr4* gene with a DNA-construct for C-terminal tagging with an HA-tag **(Figure S2A and B)**, indicating that the failure to delete the gene from blood stages is not due to inaccessibility of the locus for genetic modification. However, we were unable to detect expression of the HA-tagged MDR4 by immunofluorescence using anti-HA antibodies, possibly due to low expression levels in blood stages **(Figure S2C)**.

We were successful in obtaining mutant parasites lacking *Pbmdr2*, *Pbmdr3* and *Pbmdr5*, in which correct deletion was demonstrated by Southern analysis of separated chromosomes and diagnostic PCR for 5' and 3' TR integration **(Figure 1B)**. We also generated a mutant expressing *Pb*MDR2 tagged at the C-terminus with an mCherry-3xMyc tag (*Pbmdr2::mCherry*; **Figure 1A)**. Correct tagging was shown by diagnostic PCR for 5' and 3' TR integration of the tagging construct **(Figure 1B)**.

Northern analysis of transcription in blood stages indicates that all three genes are transcribed in blood stages **(Figure 1C)**. Also RNAseq analyses of synchronized *P. berghei* blood stages showed expression in asexual trophozoites/schizonts **(Table S2)**. No transcript could be detected in *PbΔmdr3* and *PbΔmdr5* blood stage parasites **(Figure 1C)**. We did observe a small transcript of 1.2kb in *PbΔmdr2* parasites, which encodes for the first 85 amino acids of MDR2, while the full size transcript of 4.5kb was present in WT parasites, which encodes for the protein of 948 amino acids **(Figure 1C)**.

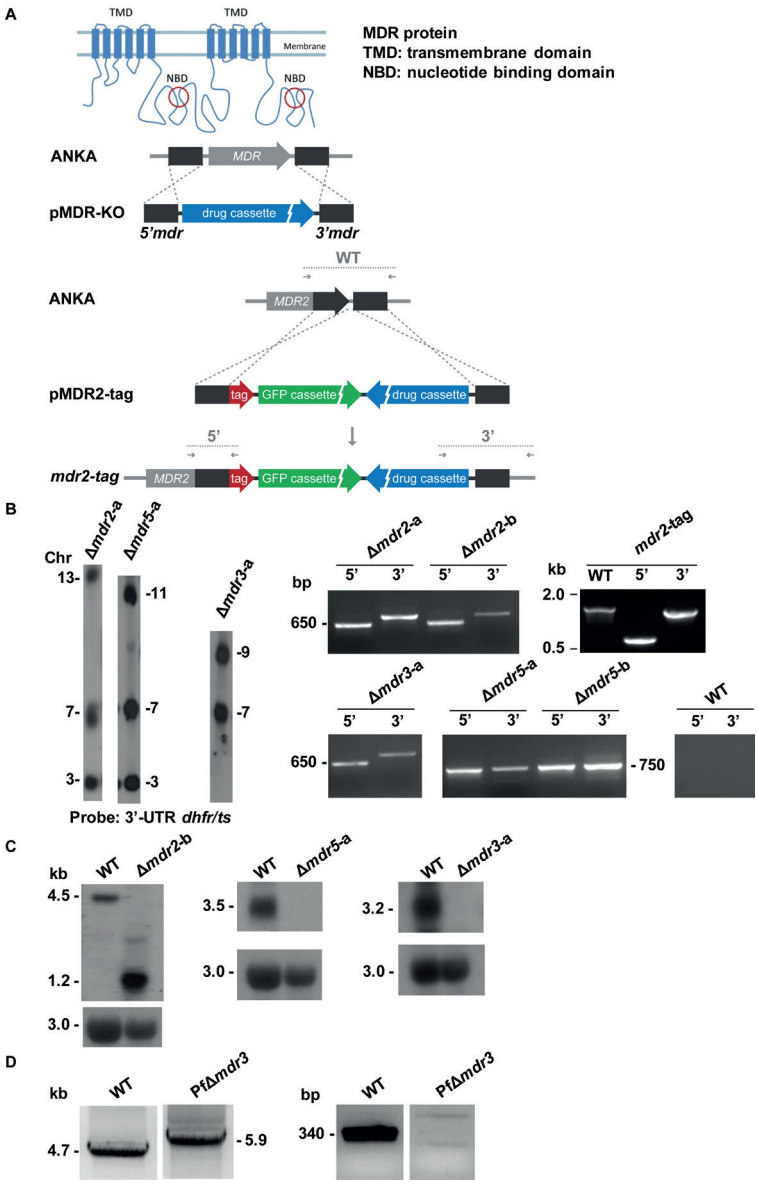


Figure 1. MDR transporters targeted for deletion in *P. berghei* and *P. falciparum*.
A Schematic overview of ABC transporter structure (top), double crossover deletion mechanism (middle) and mdr tagging strategy (bottom). **B** Southern analysis of separated chromosomes and diagnostic PCR confirms correct disruption of the mdr genes in *Δmdr2*, *Δmdr3* and Δ*mdr5* (left panel). Separated chromosomes were hybridized using a 3'UTR pbdhfr probe that recognizes the DNA-construct integrated into the *mdr2*, *mdr3*, and *mdr5* loci on chromosome 13, 9 and 11 respectively and the endogenous *dhfr/ts* on chromosome 7. In addition in *Δmdr2* and Δ*mdr5* it recognizes the GFP-luciferase reporter cassette in the *230p* locus on chromosome 3 of the parent line. Diagnostic PCR analysis showing correct integration of the gene targeting construct using primers (see **Table S4** for the primer sequences) that amplify both the 5′ and 3′ side of the integration regions after targeting *Pbmdr2* both for deletion and *mCherry-3xMyc* tagging as well as *Pbmdr3* and *Pbmdr5* for deletion(right panel). **C** Northern analysis showing the presence of transcripts of all three mdr genes in blood stages of WT parasites. In blood stages of both the *Δmdr3* and Δ*mdr5* transcripts are absent. In *Δmdr2* a small

transcript of 1.2kb is observed, which encodes for the first 85 amino acids of MDR2, while the full size transcript of 4.5kb is present in WT. **D** Diagnostic PCR using either Long Range (left panel) or intra-ORF (right panel) PCR amplification of genomic DNA from both wild type (NF54) and *PfΔmdr3* lines confirming deletion of *Pfmdr3* using specific primers **(Table S4)**, respectively. The Long Range PCR shows an increased product size in the *PfΔmdr3* line resulting from integration of the selectable marker cassette. The intra-ORF PCR amplifies the expected fragment of 340 bp in WT, whereas this fragment is absent in the mutant PCR as a result of *Pfmdr3* gene excision.

For *PbΔmdr5* parasites, asexual growth was significantly decreased resulting in a multiplication rate of 6.8 per 24 hr (SD 0.8; n = 5), which is 10 per 24 hr in WT (SD 0, n = 10)[261]. For *PbΔmdr2* the multiplication rate of was comparable to WT **(Table 1)**. For *PbΔmdr3* we have only been able to select mutants in a single transfection experiment. Both clones from this experiment showed a strongly reduced growth rate of asexual blood stages (mean multiplication rate of 4.2x per 24 hr (SD 0.6; n=2). In all other transfection experiments (n=9) targeting *Pbmdr3* we were unable to select for parasites with a disrupted *mdr3* locus, probably due to the strong reduction in growth rate. Because of the failure to select an independent secondary mutant we decided to discontinue further analysis of the *PbΔmdr3* phenotype.

Combined, these results show that *Pb*MDR2, 3 and 5 are dispensable for blood stage development of *P. berghei*, although the lack of both MDR3 and MDR5 appears to affect the growth rate of blood stage parasites.

Lines WT	**Asexual multiplication rate[1] (SD)**	**Gametocyte production[2] % (SD)**	**Ookinete production[3] % (SD)**	**Oocyst production day 12-13[4] (SD)**	**Oocyst production day 16-17[4] (SD)**	**Sporozoite production[5] X 10[3] (SD)**	**Oocyst size day 12[6] µM (SD)**	**Oocyst size day 16[6] µM (SD)**
WT								
1037m1f 1m0c1 (a)	10 (0) n=10	15-256	50-906	375 (228)	266 (117)	35 (12.4)		
676m1cl1 (b)	10 (0) n=10	15-256	50-906	224 (134)	151 (242)	58.8 (7.8)	33 (3)	33 (6)
Mutants								
Δ*mdr2*-a	10 (0) n= 4	ND	ND	152 (89.0)	17.8 (20.9)	1.2 (0.5)		
Δ*mdr2*-b	10 (0) n= 2	16.9 (1.0)	80.7 (9.1)	145 (151)	20.6(10.9)	3.5 (1.0)	19 (5)	27 (6)
Δ*mdr5*-a	6.8 (0.8) n=2	18.2 (1.4)	71.7 (6.5)	147 (102)	50.2 (29.1)	4.5 (1.3)		
Δ*mdr5*-b	6.7 (0.7) n=3	ND	ND	168 (169)	94.5 (26.0)	9.8 (2.9)	21 (2)	33 (3)

Table 1: Phenotypes of *P. berghei* mutants lacking expression of MDR proteins
[1] The multiplication rate per 24 hour of blood stage parasites in mice infected with a single parasite;
[2] The percentage of blood stage parasites developing into gametocytes *in vivo*;
[3] The percentage of female gametes developing into mature ookinetes *in vitro*;
[4] The mean number of oocysts per mosquito
[5] The mean number of salivary gland sporozoites per mosquito (day 19–22);
[6] The developmental data for wild type parasites are shown as the range of mean values of > 10 experiments.
ND, not determined.

Dispensable roles of *P. falciparum* MDR2, MDR3, and MDR5 for blood stage development

We have previously generated *P. falciparum* mutants lacking expression of MDR2 and MDR5[265]. Generation of these mutants indicate that both MDR2 and MDR5 are dispensable for blood stages and confirms the observations in *P. berghei*. In this study we additionally targeted *mdr3* and *mdr6* for deletion with the pHHT-FRT-(GFP)-*Pfmdr3* and pHHT-FRT-(GFP)-*Pfmdr6* deletion constructs **(Figure S1)** [151,266]. In agreement with our findings in *P. berghei*, we were unable to isolate *P. falciparum* parasite mutants lacking *mdr6* in three transfection experiments (data not shown), which indicates an essential role of the MDR6 protein for blood stage development. In contrast, we were able to readily select mutants lacking expression of MDR3. Correct deletion of *Pfmdr3* was validated using a diagnostic LR-PCR in which the expected larger amplification product (including selectable marker cassette) was obtained for parasites lacking this gene compared to WT **(Figure 1D)**. In addition, an intra-exonic PCR confirmed absence of the *Pfmdr3* gene in the mutant line **(Figure 1D)**.

Our ability to generate *P. falciparum mdr2*, *mdr3*, and *mdr5* mutants indicates dispensable roles during blood stage development. We next analyzed the *in vitro* growth rate of *P. falciparum* blood stages over a period of 7 days using a modified pLDH method[268]. In this assay, mutants lacking MDR2, MDR3 and MDR5 showed a normal growth rate, whereas the maximal parasitemia that was reached in cultures of *PfΔmdr2* parasites was significantly lower at day 7 compared to WT parasites of the NF54 strain **(Figure 2)**.

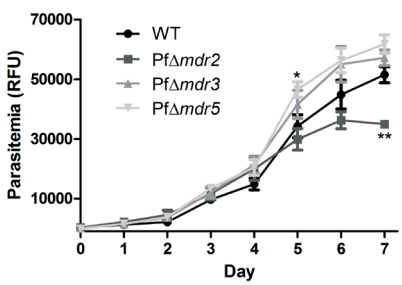


Figure 2. Reduced maximal parasitemia in *PfΔmdr2* parasites.
Growth of asexual blood stages of WT and two clonal lines for *PfΔmdr2*, *PfΔmdr3* and *PfΔmdr5* (data of two clones per mutant was pooled) was monitored over a 7-day period in the in vitro multiplication assay. In vitro parasite cultures were started at 0.1% parasitemia and growth was determined by measuring relative fluorescence units (RFU) in three independent experiments (triplicate samples per time point) using a modified pLDH method[268]. Asexual multiplication was comparable for *mdr3* and *mdr5*, however, maximal parasitemia of *PfΔmdr2* was significantly reduced at day 7.

MDR2 and MDR5 of *P. berghei* play a role during mosquito stage sporogony

We explored the phenotypes of *PbΔmdr2* and *PbΔmdr5* during sexual, mosquito and liver stages. The gametocyte and ookinete conversion rate resembled that of WT parasites **(Table 1)**. Whereas oocysts were readily detected in mosquito midguts that were fed on mice infected with *P. berghei* parasites lacking expression of MDR2 or MDR5, the subsequent sporogonic development was significantly affected. The mean number of *PbΔmdr2*-b and *PbΔmdr5*-b GFP-positive oocysts was 48 ± 13 and 63 ± 16, respectively, between day 9-23 after mosquito feeding **(Figure 3A)** and was significantly reduced compared to WT (128 ± 24; $p < 0.05$). Moreover, this effect was increased in time as at later time points (day 16-17) fewer oocysts could be detected for both lines when compared to earlier time points (day 12-13) **(Table 1)**. Many of the MDR2 and MDR5 deficient oocysts appeared immature **(Figure 3E)** and their average size was significantly smaller at day 12 compared to WT **(Table 1)**. However, at day 16, mutant oocysts measured similar sizes as WT oocysts, suggesting that the lack of the MDR2 or MDR5 protein resulted in a delayed growth **(Table 1)**. Maturation of oocysts could be specifically monitored in the 1037m1f1m0cl1 parent line. This reference line expresses the reporter fusion protein GFP-Luciferase under the control of the *ama-1* promoter. In mosquito stages, this promoter is only active in mature oocysts when sporozoites are formed. We therefore counted GFP-positive oocysts in mutant and WT-infected mosquitoes at day 17 after infection. Developmental retardation was evident and significant for the *PbΔmdr2*-a and *PbΔmdr5*-a gene deleted parasites as only 1.0% (SD 1.6%) and 23% (SD 11%) of the oocysts in the respective deletion mutants reached full maturation as shown by GFP expression, compared to 82% (SD 9.7%) in WT **(Figure 3F)**. This also resulted in strongly reduced numbers of sporozoites. Only 3.4% - 6.0% and 13 - 17% of WT sporozoite numbers could be isolated from salivary glands of *PbΔmdr2* and *PbΔmdr5* infected mosquitoes, respectively **(Table 1; Figure 3B)**.

These results indicate that both MDR2 and MDR5 play a role during sporogony. No published data is available on expression of these proteins in *P. berghei* oocysts or sporozoites, although evidence has been reported for transcription of MDR5 in sporozoites **(Table S1)**. Moreover, *P. falciparum* MDR5 has been found in proteomes of sporozoites. MDR2 has only been detected in proteomes of *P. falciparum* asexual blood stages and gametocytes. We analysed expression of *Pb*MDR2 in blood stages, oocysts and sporozoites of the mutant *Pbmdr2*::mCherry by fluorescence microscopy. In both asexual blood stages and gametocytes fluorescence signals were detected, which is in agreement with MDR2 detection in the *P. falciparum* proteomes of these stages. The fluorescence signals are mainly associated with hemozoin granules (in both asexual stages and gametocyte;

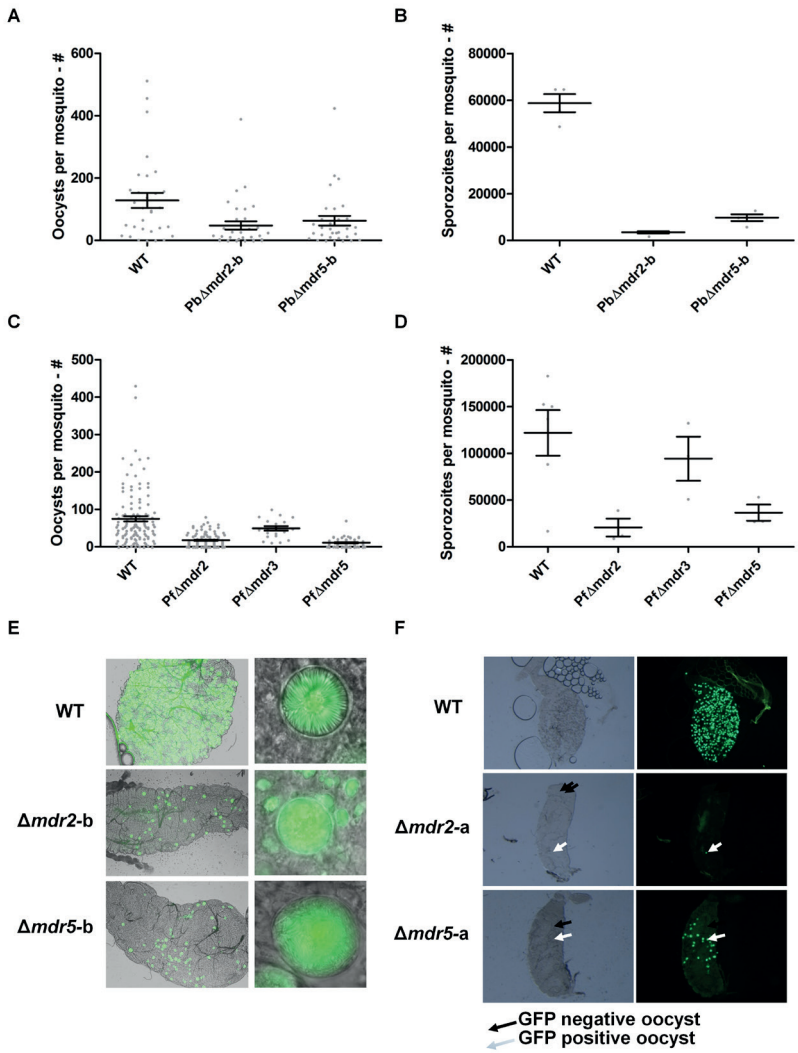


Figure 3. Altered mosquito stage development of mutants lacking expression of MDR2 and MDR5.
A Oocyst production in *A. stephensi* mosquitoes of *P. berghei* parasites lacking MDR2 and MDR5 is significantly reduced ($p < 0.05$). **B** Sporozoite production in *A. stephensi* mosquitoes of *P. berghei* parasites lacking MDR2 and MDR5 is significantly reduced ($p < 0.0001$) **C** Following *P. falciparum* standard membrane feeding assays using female *A. stephensi* mosquitoes, oocysts were counted at day 7 post infection in wild type NF54, *PfΔmdr2*, *PfΔmdr3* and *PfΔmdr5* parasites. Oocyst production of parasites lacking MDR2 and MDR5 expression was significantly reduced ($p < 0.0001$), however, oocyst production of *PfΔmdr3* was unaffected ($p > 0.05$). **D** The number of sporozoites that could be isolated from the salivary glands of the infected mosquitoes was decreased for *PfΔmdr2* ($p < 0.05$) and *PfΔmdr5* ($p = 0.05$), but not for *PfΔmdr3* ($p > 0.05$) parasites isolated at day 14-16 or 18 post infection. Each data point represents the average number of sporozoites from 10 dissected mosquitoes in an experiment. **E** Oocyst number and maturity was substantially decreased at day 16 in *P. berghei* gene deletion lines compared to WT, as shown by GFP positivity in whole midguts and sporogony per oocyst in phase-contrast microscopy. **F** Strongly decreased formation of mature oocysts in *PbΔmdr2* and *PbΔmdr5* as visualized by mature GFP-expressing oocyst. Both WT and mutant parasites express GFP under control of the *ama1* promotor that is only active in mature oocysts that undergo sporogony.

Figure 4A), which may suggest that this protein is located on the food vacuole membrane as *P. berghei* trophozoites and gametocytes have many small food vacuoles. In mature schizonts these food vacuoles merge into one or two large vesicles containing hemozoin, and it is in these vesicles that we also observe fluorescent signal; **Figure 4A)**. In addition, we found fluorescence signals associated with the plasma lemma of blood stages, which is especially clear in merozoites of mature schizonts.

We also observed fluorescence signals during oocyst development **(Figure 4B)**. From day 10 onwards, before sporozoite formation, we observe a highly structured fluorescence pattern within the oocyst possibly associated with early sporoblast formation[269] In more mature oocysts, when sporozoite formation is observed, the fluorescence signal becomes more diffuse but it is only associated with the areas within the oocyst where sporozoites are present and are budding from the sporoblasts.

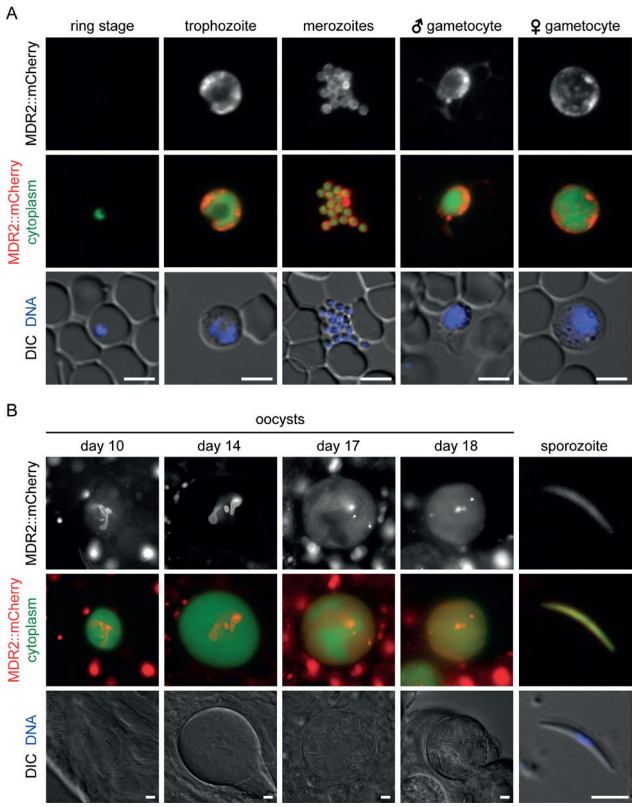


Figure 4. Localization of *Pb*MDR2::mCherry
Expression of MDR2::mCherry during *P. berghei* blood (A) and mosquito (B) stage development. Upper row represents mCherry expression, middle row is combined expression of cytoplasmic GFP (green) and MDR2::mCherry (red), lower row shows phase-contrast and nuclear Hoechst33342 (blue) staining. **A** Shown are parasites during ring, trophozoite, merozoite and male as well as female gametocyte stages. **B** Shown are oocysts 10, 14, 17 and 18 days after the mosquito bloodmeal and a salivary gland-associated sporozoite.

Infectivity of *PbΔmdr2* or *PbΔmdr5* salivary gland sporozoites was tested by intravenous injection of 10,000 parasites into mice. All mice (n=3) developed blood stage infection after a prepatent period of 5-6 days which is comparable to the prepatent period of WT sporozoites[215].

In conclusion, *Pb*MDR2 and *Pb*MDR5 play an important role in both oocyst formation and maturation as well as sporozoite development. In the mutants lacking these proteins, lower total numbers of oocysts were observed, and in addition to a lower percentage of fully mature oocysts, this resulted in strongly reduced sporozoite formation. No evidence was found that sporozoites that were produced in parasites lacking MDR2 or MDR5 expression had a lower infectivity to mice.

***P. falciparum* MDR2 and MDR5 play a role during mosquito stage sporogony**

Oocyst and sporozoite formation was also analyzed in the *P. falciparum* mutants *PfΔmdr2*, *PfΔmdr3*, and *PfΔmdr5*. Gametocytes of these parasites were fed to *Anopheles stephensi* mosquitoes using a standard membrane feeding assay[153]. First, oocysts in mosquito midguts were quantified 7 days post infection in 1 to 7 independent feeding experiments. Oocyst production in *PfΔmdr3*-infected mosquitoes (50 ± 6 oocysts; n=1, 20 mosquitoes) was not significantly different ($p > 0.05$) from wild type infected mosquitoes (75 ± 7 oocysts; n=7, 120 mosquitoes). This is in contrast with *PbΔmdr2* and *PbΔmdr5* infected mosquitoes, where the number of oocysts was significantly reduced (both $p < 0.0001$) to only 18 ± 2 oocysts (n=6, 120 mosquitoes) and 11 ± 2 oocysts (n=4, 60 mosquitoes), respectively **(Figure 3C)**. Sporozoite formation was analyzed by determining the mean in 10 mosquitoes per experiment by salivary gland dissection at 14-16 or 18 days post infection. In infected mosquitoes, the number of salivary gland sporozoites was significantly reduced to only 17.2% ($p < 0.05$) for *PfΔmdr2* (n=3) and borderline significant ($p = 0.05$) to 30.3% for *PfΔmdr5* (n=3) compared to NF54 WT (n=6) **(Figure 3D)**. In contrast, mosquitoes fed on *PfΔmdr3* (n=3) had wild type levels of salivary gland sporozoites **(Figure 3D)**.

DISCUSSION

In this study, we analyzed the role of seven MDR proteins during the life cycle of *P. berghei* and *P. falciparum* malaria parasites using reverse genetic methods. As a first screen, all seven genes were targeted for deletion in the *P. berghei* rodent model since for this parasite highly efficient and standardized methods exist for deletion of genes by double cross-over homologous constructs using linear DNA constructs. Despite multiple attempts to delete the *mdr1*, *mdr4*, *mdr6* and *mdr7*

genes from the *P. berghei* genome, we could not select gene deletion mutant parasites. These results suggest that these genes are essential for blood stage multiplication. In addition, the unsuccessful attempts to delete the genes encoding for MDR1[270] and MDR6 (this study) in *P. falciparum* supports our findings in *P. berghei* that these proteins are essential for blood stage development of malaria parasites. We therefore focused on the function of the *mdr2*, *mdr3*, and *mdr5* genes that could be deleted from the *P. berghei* genome and generated equivalent mutants in *P. falciparum*.

Mdr2, *mdr3*, and *mdr5* were successfully deleted in *P. berghei* and *P. falciparum*. In *P. berghei*, data from Northern analysis (this paper) and RNAseq analysis [271] indicated that these three genes are transcribed in blood stages, suggesting that they play a role during blood stage development. Also by analyzing a mutant expressing a mCherry-tagged version of MDR2 we found expression in asexual blood stages and in gametocytes. The fluorescence signals were associated both with hemozoin crystals and with the surface of parasites suggesting a location in both the membrane of food vacuoles surrounding the hemozoin crystals and in the plasma lemma membrane. Although the ability to delete these genes may indicate that other proteins compensate for the loss MDR transporter function, the reduced growth we observed for *P. berghei* blood stages lacking expression of MDR3 and MDR5 does suggest a role of these proteins in these stages. The lack of a growth phenotype in blood stages of the *P. berghei* mutant lacking MDR2 suggests either the absence of a function in blood stages or complete compensation of its role by other proteins. Also for the *P. falciparum* genes evidence is present for blood stage expression, both at the transcript and protein level **(Table S2)**. However, and in contrast to the *P. berghei* results, we did not find evidence for a decreased growth rate for the *Pfmdr3* and *Pfmdr5* gene deletion mutants, whereas the maximal parasitemia that was reached in cultures of *PfΔmdr2* parasites was significantly lower at day 7 compared to WT parasites. This may indicate that the function of the orthologous MDR proteins differ between blood stages of *P. berghei* and *P. falciparum* or that in *P. falciparum* blood stages the loss of the MDR3 and MDR5 proteins can be completely compensated by other proteins, whereas in *P. berghei* blood stages only the function of MDR2 can be fully compensated by (an)other protein(s). However, these discrepancies may also be due to different assays used for analysis of blood stage growth. For *P. berghei*, blood stage growth was determined *in vivo* whereas *P. falciparum* growth was analyzed under *in vitro* conditions. Although here we describe *Pfmdr2* as a dispensable gene in vitro, the T484I polymorphism is identified as a genetic background marker for kelch13 mutations[258], highlighting the possible importance of this gene for parasite survival within artemisinin-treated hosts.

Further analysis of the mutants lacking expression of MDR2 and MDR5 demonstrated that these proteins play a role in the formation of oocysts and sporozoites in both *P. berghei* and *P. falciparum*. Whereas *P. falciparum* MDR5 expression has been shown previously by analysing proteomes of sporozoites **(Table S2)**, MDR2 expression had not been reported in mosquito stages. MDR2 had only been detected in proteomes of *P. falciparum* asexual blood stages and gametocytes. By analyzing the *Pbmdr2*::mCherry mutant, we show here for the first time expression of MDR2 in oocysts and in sporozoites. Expression in these stages is in agreement with our observations of reduced sporogony in mutants lacking expression of MDR2. Absence of these proteins results in strongly reduced oocyst and sporozoite (except for *PfΔmdr5*) formation compared to WT parasites. Whereas in *P. berghei* we found a more pronounced reduction of *PbΔmdr2* compared to *PbΔmdr5* oocyst numbers, in *P. falciparum* the effect was more severe in mutants lacking MDR5 expression. The effect of MDR2 or MDR5 absence on *P. berghei* maturation was most clearly visualized in the parasite mutants where only fully mature oocysts express GFP, as a strong and significant reduction in GFP-positive oocysts was observed compared to the parent line. In both *P. berghei* and *P. falciparum*, the absence of MDR2 and MDR5 did not result in a complete block of sporozoite formation. We demonstrated that *P. berghei* sporozoites lacking either MDR2 or MDR5 were infectious to mice, resulting in blood stage infections with prepatent periods that were comparable to WT sporozoites. These observations suggest that these proteins have no function during sporozoite invasion of hepatocytes or during subsequent liver stage development.

ABC transport proteins play an important role in maintaining homeostasis in many organisms, and could be of special importance for parasites interacting with their host cell environment from which they require essential nutrients and where they dispose toxic waste products. Identification of the vital role of these transporters in different stages of the parasite life cycle may reveal novel drug targets for inhibition of parasite development and transmission prevention. In this study, we present evidence that *P. berghei mdr1*, *4, 6, 7*, and *P. falciparum mdr6* are likely to be essential for asexual multiplication, highlighting the potential of these transporters or their substrates as drug targets for treating blood stage malaria. Furthermore, *mdr2* and *5* play a significant role in mosquito-stage development in both species. Further unraveling their involvement in physiological pathways could lead to novel strategies to target parasite transmission, and the determination of substrate specificity of these transporters is therefore an essential next step.

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Unsuccessful attempts	**Gene ID**	***P. falciparum* orthologue**	**DNA construct name**	**Experiment No1**	**Parent line2**	**RMgmDB ID3**
mdr1	PBANKA_123780	PF3D7_0523000	pL1617	1624, 1656, 1724	1037m1f1cl1 676m1cl1	RMgm-1158
mdr4	PBANKA_040120	PF3D7_0302600	pL1725	1820, 1881, 1929, 2040	1037m1f1cl1	RMgm-1159
mdr6	PBANKA_136480	PF3D7_1352100	pL1923	2128, 2144, 2145, 2160	1037m1f1cl1 676m1cl1	RMgm-1160
mdr7	PBANKA_060830	PF3D7_1209900	pL1629	1665, 2103, 1728	1037m1f1cl1 676m1cl1	RMgm-1161
Mutants	**Gene ID**	***P. falciparum* orthologue**	**DNA construct name**	**Experiment No1**	**Parent line2**	**RMgmDB ID3**
Δmdr2-a	PBANKA_131170	PF3D7_1447900	pL1724	1928cl3	1037m1f1cl1	RMgm-1165
Δmdr2-b				2135cl1	676m1cl1	RMgm-1166
Δmdr5-a	PBANKA_135330	PF3D7_1339900	pL1726	2041cl1	1037m1f1cl1	RMgm-1168
Δmdr5-b				2137cl1	676m1cl1	RMgm-1169
Δmdr3	PBANKA_090350	PF3D7_1145500	pL1985	2308cl1	1037m1f1cl1	RMgm-1167

Table S1: Targeting *P. berghei* MDR genes for deletion: unsuccessful attempts and mutants generated

[1] Experiment number for independent transfection experiments: the unsuccessful attempts and the experiment number/ clone of the gene deletion mutants

[2] Parent *P. berghei* ANKA line in which the genes were targetted for deletion

[3] The ID number of the mutants (or of the unsuccessful attempts for gene deletion) in the RMgm database (www.pberghei.eu) of genetically modified rodent malaria parasites

Protein	Gene ID	mRNA				Protein			
P. berghei		As1	Gct1	Ook2	Sp2	As3	Gct4		
MDR1	PBANKA_123780	++	++	+	-	+	++		
MDR2	PBANKA_131170	+	-	-	-	+/-	-		
MDR3	PBANKA_090350	+	-	-	-	-	-		
MDR4	PBANKA_040120	+	-	-	-	-	-		
MDR5	PBANKA_135330	+	+	-	+/-	-	-		
MDR6	PBANKA_136480	+	-	+	+/-	-	-		
MDR7	PBANKA_060830	+	+	-	-	-	-		
P. falciparum		As5	Gct5		Sp6	As3	Gct3	Ooc3	Sp3
MDR1	PF3D7_0523000	++	++		+/-	++	++	-	-
MDR2	PF3D7_1447900	+/-	+/-		+/-	++	++	-	-
MDR3	PF3D7_1145500	+/-	+/-		+/-	+/-	-	-	-
MDR4	PF3D7_0302600	+/-	+/-		+/-	+/-	+/-	-	+/-
MDR5	PF3D7_1339900	+/-	+/-		+/-	+/-	+	-	++
MDR6	PF3D7_1352100	+	+/-		+/-	-	+/-	-	-
MDR7	PF3D7_1209900	+	+/-		+/-	-	+	-	-

Table S2. Expression profile of *Plasmodium mdr* genes

AS, asexual stages; Gct, gametocytes; Ook, ookinetes; Ooc, oocysts; Sp, sporozoites

[1] Rodent malaria gene expression (+ = 21-100 ; ++ >100)[272]

[2] PlasmoDB EST data (+/- = 1-5 ESTs)

[3] PlasmoDB proteome data (+/- = 1-2; + = 3-10; ++ > 10)

[4] PbANKA Male vs female gametocyte proteome [273]

[5] PlasmoDB RNAseq (FPKM) data (+/- < 10; + = 10-20; ++ > 20)

[6] PlasmoDB oligo array (+/- = 1-10) RMA value (log2)

	Construct	Basic construct	#	Sequences	Restriction sites	Description
Gene deletion constructs						
Pbmdr1	pL1617	pL0001	1	GGGGGTACCATTTTTT-GAATGGTGCTTCC	*Asp718I*	5′- *mdr1* targeting region F
			2	GGGAAGCTTCTCTTTC-TCAACTTCATGTTTG	*HindIII*	5′- *mdr1* targeting region R
			3	GGGGAATTCCTGTTTAC-AAAAATAGTGGC	*EcorI*	3′-m*dr1* targeting region F
			4	GGGTCTAGACGGGGTAT-GTATCTGTAAATTTTC	*XbaI*	3′- *mdr1* targeting region R
Pbmdr2	pL1724	pL0001	5	GGGGGTACCGGGTATA-ATATATATTCGTC	*Asp718I*	5′- *mdr2* targeting region F
			6	GGGAAGCTTCCGAAA-GTGTTGTATATCC	*HindIII*	5′- *mdr2* targeting region R
			7	GGGGAATTCGTACTAT-ATCCTCAGCAGAATC	*EcorI*	3′- *mdr2* targeting region F
			8	GGGTCTAGACGGTACG-CATATACTCAGG	*XbaI*	3′- *mdr2* targeting region R
Pbmdr3	pL1985	pL0001	9	GGGGGTACCCTTTTTG-TGCAATTATAATGGG	*Asp718I*	5′- *mdr3* targeting region F
			10	GGGAAGCTTCCTCATA-TTCAATGCTACAACC	*HindIII*	5′- *mdr3* targeting region R
			11	GGGGAATTCCTTTACA-GATGGGTATTTG	*EcorI*	3′- *mdr3* targeting region F
			12	GGGTCTAGACCAAAAT-TCGATCATAACAC	*XbaI*	3′- *mdr3* targeting region R
Pbmdr4	pL1725	pL0001	13	GGGGGTACCGTTGCTTAT-CGTATTATGGG	*Asp718I*	5′- *mdr4* targeting region F
			14	GGGAAGCTTCCCAG-CAGTTTCATGTTTC	*HindIII*	5′- *mdr4* targeting region R
			15	GGGGAATTCCCACA-ATTAAGCATTGTG	*EcorI*	3′- *mdr4* targeting region F

	Construct	Basic construct	#	Sequences	Restriction sites	Description
			16	GGGTCTAGAGCATAT-GTACACACATACATGTG	*XbaI*	3′- M*mdr4* targeting region R
Pbmdr5	pL1726	pL0001	17	GGGGGTACCATGTC-TCTCATTTTTGAG	*Asp718I*	5′- *mdr5* targeting region F
			18	GGGAAGCTTCTTAC-AAAGGTTATTCCC	*HindIII*	5′- *mdr5* targeting region R
			19	GGGGAATTCCAATG-TCTTCTGGACAGAAAC	*EcorI*	3′- *mdr5* targeting region F
			20	GGGTCTAGAGTGGAT-GTTGCATATGTATGG	*XbaI*	3′- *mdr5* targeting region R
Pbmdr6	pL1923	pL0001	21	GGGGGTACCATTTTT-TGAATGGTGCTTCC	*Asp718I*	5′- *mdr6* targeting region F
			22	GGGAAGCTTGGTGT-GATATTTTTGAAC	*HindIII*	5′- *mdr6* targeting region R
			23	GGGGAATTCGCAAA-CATATTTCATAAATTATCC	*EcorI*	3′- *mdr6* targeting region F
			24	GGGTCTAGACACAAA-AAATATGGAATGCTCG	*XbaI*	3′- *mdr6* targeting region R
Pbmdr7	pL1629	pL0001	25		*Asp718I*	5′- *mdr7* targeting region F
			26	GGGAAGCTTGCTAC-TAAGCATGCGAAGC	*HindIII*	5′- *mdr7* targeting region R
			27	GGGGAATTCGGATCA-TTTTTATCGGGTGGTC	*EcorI*	3′- *mdr7* targeting region F
			28	GGGTCTAGAGTGC-ATATATACATGTACAC	*XbaI*	3′- *mdr7* targeting region R
Pfmdr3	pHHT-FRT-(GFP)-Pf*mdr3*	pHHT-FRT-(GFP)-Pf52	29	GGGGCGCGCCTTT-TCATGAATTTAAAGCC	*BssHII*	5′- *mdr3* targeting region F
	pHHT-FRT-(GFP)-Pf*mdr3*	pHHT-FRT-(GFP)-Pf52	30	GGGCGTACGTCTC-TTAAAAGGATGAGG	*BsiWI*	5′- *mdr3* targeting region R
	pHHT-FRT-(GFP)-Pf*mdr3*	pHHT-FRT-(GFP)-Pf52	31	GGGCCCGGGTCGA-ATGGATTAACAAATG	*XmaI*	3′- *mdr3* targeting region F

	Construct	Basic construct	#	Sequences	Restriction sites	Description
	pHHT-FRT-(GFP)-Pf*mdr3*	pHHT-FRT-(GFP)-Pf52	32	GGGGCTAGCGTCT-CGTTAAATAACCATC	*NheI*	3′- *mdr3* targeting region R
Pfmdr6	pHHT-FRT-(GFP)-Pf*mdr6*	pHHT-FRT-(GFP)-Pf52	33	GGGGCGCGCTTTCC-AATGAGCCAACG	*BssHII*	5′- *mdr6* targeting region F
	pHHT-FRT-(GFP)-Pf*mdr6*	pHHT-FRT-(GFP)-Pf52	34	GGGCGTACGCATTT-ATTATAAAAACTGCGC	*BsiWI*	5′- *mdr6* targeting region R
	pHHT-FRT-(GFP)-Pf*mdr6*	pHHT-FRT-(GFP)-Pf52	35	GGGCCCGGGCGTCC-TAGATGAACATGCAAG	*XmaI*	3′- *mdr6* targeting region F
	pHHT-FRT-(GFP)-Pf*mdr6*	pHHT-FRT-(GFP)-Pf52	36	GGGGCTAGCGAAATG-ATATGTACATTATACAGG	NheI	3′- *mdr6* targeting region R

	Construct	Basic construct	#	Sequences	Restriction sites	Description
Gene tagging constructs						
Pbmdr2	pMTP-IM-Pb*mdr2*	pBAT-SIL6	37	AATCCTAGGTTTGGAAAGT-AATCCAAATACTAATCG	*AvrII*	3′- *Mdr2* insert F
			38	ATAGGTACCATAATTGCTTT-ATTTCATATATCGATGG	*KpnI*	3′- *Mdr2* insert R
			39	AATCCGCGGCTCAAGATAC-TATATTATTTAATGAAACG	*SacII*	C terminus insert F
			40	AAAGAATTCAATATCGTTAT-TACCAGATTGCATATT	*EcoRI*	C terminus insert R

Table S3 Targeting constructs and primers

Gene	No.	Sequences	Description
Pb diagnostic primers			
Pbmdr1	41	CCTATACATGTATAACTAC	*Mdr1* 5′- in F
	42	TAGTCAGAATTCAAGGGTG	*Mdr1* 3′- in R
Pbmdr2	43	GGTTGATATATAAAGGTAGAG	*Mdr2* 5′- in F
	44	GGATACAATGCTGTATCTTC	*Mdr2* 3′- in R
Pbmdr2::mCherry-3xMyc	45	CTGAAGGAGATATTAAAATAGGAGG	*Mdr2* 5′ F
	46	CCCTCCATGTGAACCTTGAAG	mCherry R
	47	ATGAAATACCGCTCCATTTTTCC	5′ DHFR F
	48	CAAAAAATCTGGATATATTATTTATACATGC	*Mdr2* 3′ R
Pbmdr3	49	GAAGGGATGTTATACACG	*Mdr3* 5′- in F
	50	CTGTACCTGTATTTGGAG	*Mdr3* 3′- in R
Pbmdr4	51	GTATATTACATACATGCAC	MDR4 5′- in F

Gene	No.	Sequences	Description
	52	GTATTATTTGCGCAGGCATG	MDR4 3′- in R
Pbmdr5	53	GTGCATGATATATATAAGTGTG	*Mdr5* 5′- in F
	54	GTTGAAAAGGGAAAGATGAG	*Mdr5* 3′- in R
Pbmdr6	55	GCATATGTAGAAGCTTGC	*Mdr6* 5′- in F
	56	GATGTGATCGGAAGGCCATC	*Mdr6* 3′- in R
Pbmdr7	57	GGGTGGAGGTTTTAAAGAGG	*Mdr7* 5′- in F
	58	TGCTCATAATGTGTTTATCC	*Mdr7* 3′- in R
Pb SM primers			
5′ in R	59	TTGACCTGCAGGCATGCAAGC	*Mdr1* ORF, F
3′ in F	60	GATTCATAAATAGTTGGAC	*Mdr1* ORF, R
Pf diagnostic primers			
Pfmdr3	61	CTTTTCATGAATTTAAAGCC	*Mdr3* 5′- long range F
Pfmdr3	62	AAGAAGACTTATCCGATATG	*Mdr3* 3′- long range R
Pfmdr3	63	TGTATAGTACCGAATTACATGAATG	*Mdr3* ORF F
Pfmdr3	64	CTCGTAAATCTGCCGCTTATATG	*Mdr3* ORF R

Table S4 Analytical primers

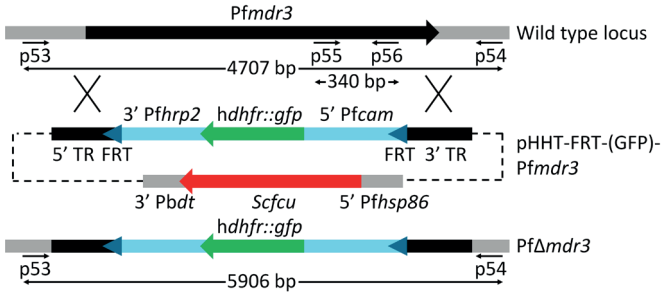

Figure S1. Schematic of the strategy of *Pfmdr3* deletion and genotyping of the *PfΔmdr3* mutant.
Upon transfection of the HHT-FRT-(GFP)-Pf*mdr3* construct and positive selection with WR99210, mutants are selected in which the construct integrates into either the 5′ or 3′ target region (TR) in the wild type locus. Subsequent negative selection on 5-fluorocytosine selects for *mdr* gene replacement by the positive selectable marker cassette harboring the *hdhfr::gfp* fusion gene flanked by FRT sites (blue triangles). This results in selection of parasites with the *Pfmdr3* gene deleted (*PfΔmdr3*). *cam*: calmodulin; *hdhfr::gfp*: human dihydrofolate reductase fused to green fluorescent protein; *hrp*: histidine rich protein; *hsp*: heat shock protein; *mdr*: multidrug resistance; *Scfcu*: *Saccharomyces cerevisiae* cytosine deaminase/uracil phosphoribosyl-transferase; *Pbdt*: *P. berghei dhfr* terminator; FRT: flippase recognition target; p: primer; TR: target region.

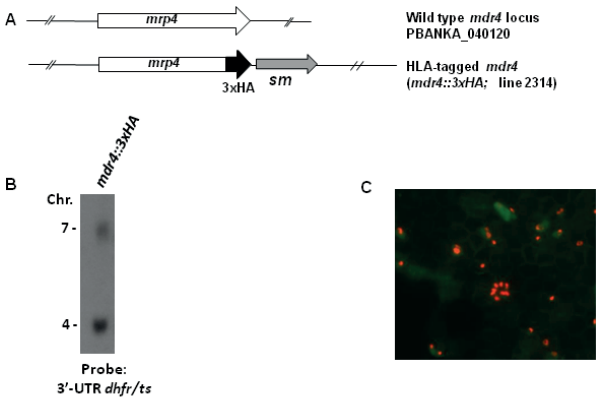

Figure S2. *Pbmdr4* tagging and lo calization
A The *mrp* gene was C-terminally tagged with a 3xHA-tag using a PlasmoGem DNA construct (PbGEM-084058; pL1995) that integrates by double cross-over integration and contains the *hdhfr/yfcu* selectable marker (sm). **B** Genotyping of mdr4::3xHA by Southern analysis of separated chromosomes (chr.) and diagnostic PCR. Hybridisation with the 3'-UTR *dhfr/ts* probe recognizes the endogenous *P. berghei dhfr/ts* gene on chr. 7 and the integrated DNA construct in the *mdr4* locus on chr. 4. **C** Expression of MDR4::3xHA was shown by IFA analysis of blood stage parasites using anti-HA antibodies and Alexa488 (green) and DAPI nuclear staining (red).

CHAPTER 6

Discussion

INTRODUCTION

ATP-binding cassette (ABC) transporters are evolutionary well-conserved membrane proteins that are involved in cellular multidrug resistance. They actively extrude xenobiotics, which results in decreased drug concentrations at target sites in many organisms, including the malaria parasite *Plasmodium falciparum*. As their function in *Plasmodium* species remained a rather unexplored territory, we aimed to study all ABC transport proteins in *P. falciparum* as well as the rodent model organism *P. berghei*. In the latter we targeted all 9 ABC transport genes[148] for deletion and obtained mutant parasites for *Pbmdr2*, *Pbmdr3*, *Pbmdr5* and *Pbabcg1*. After multiple ineffectual attempts to obtain viable parasites after removal of *Pbmdr1*, *Pb*mdr4, *Pbmdr6* and *Pbmdr7*, we concluded that these genes are essential for blood stage multiplication and parasite survival. The deletion of *Pbmrp* orthologues *Pfmrp1* and *Pfmrp2* was subsequently performed in *P. falciparum*. As the parasite has to undergo complete transformation more than once during its life cycle, adaptations of *Plasmodium* to very diverse environments is required. Therefore, we did not only study the phenotype in asexual stages, but also during mosquito- and liver-stage development.

Interaction of antimalarial compounds with human ABC transporters

Human ABC transport proteins may affect antimalarial drug effectivity through alterations of local drug concentrations and drug-drug interactions with co-medication at the transporter site. Therefore we studied the interaction of chloroquine, quinine, artemisinin, mefloquine, lumefantrine, atovaquone, dihydroartemisinin and proguanil with transport activity of P-glycoprotein (P-gp/MDR1), breast cancer resistance protein (BCRP/ABCG2), bile salt export pump (BSEP/ABCB) and multidrug resistance-associated proteins (MRP/ABCC) 1–4 was assessed in **Chapter 2**, using a vesicular transporter overexpression assay. The strong inhibitory effect of quinine and mefloquine on P-gp-mediated transport and of mefloquine, atovaquone and proguanil on that of BCRP indicate that indeed these export proteins may be involved in drug-drug interaction with substrates of these transporters. For both atovaquone and quinine, significant inhibition of BCRP and P-gp, respectively, was observed at concentrations that are pharmacologically relevant during drug treatment regimens. Indeed, reports of suspected clinical interactions with atovaquone included azithromycin, etravirine, saquinavir, efavirenz, lopinavir/ritonavir and atazinavir/ritonavir co-mediaction[111,112,121]. Other compounds that are known to interact with BCRP-mediated transport are fluoroquinolone antibiotics, kinase inhibitors, cytostatics, antifolates, and statins[123-129]. These

drugs are not widely used in malaria-endemic areas, however, interactions with prophylactic doses of atovaquone used by travelers can be anticipated. Interactions with quinine have been described for ritonavir/lopinavir combination therapy as well as ritonavir monotherapy, and for nevirapine, rifampicin, lopinavir, cyclosporine, and digoxin[134-136,139-141]. Although quinine is not used any more in first-line treatment strategies, its use has increased as it is often applied as an alternative treatment after artemisinin-based combination therapy (ACT) stock-outs[145] and for treatment of malaria infections in pregnant women[146]. Adherence to this compound is known to be low due to frequently occurring side effects, which are often plasma concentration-dependent[147]. For these reasons, establishing effective but non-toxic plasma concentrations is essential, and interaction with co-administered compounds that mediate P-gp transport should be monitored. Especially the interaction of both atovaquone and quinine with antiretroviral medication could have severe implications on treatment strategies for both infections, as HIV is another major health issue in sub-Saharan regions of Africa[99]. Many different antiretroviral compounds are being prescribed, depending on personal characteristics and resistance status, and interactions with BCRP have been described for many of these compounds. Drug-drug interactions of atovaquone and quinine with antiretroviral medication could have severe implications on treatment strategies; unperceived decreased concentrations due to alterations in transport efficiency can lead to earlier antiretroviral therapy treatment failure or parasite recrudescence, and could have a large impact on morbidity.

The contribution of ABC transporters to *Plasmodium* drug sensitivity

In order to study the contribution of ABC transport proteins of *Plasmodium* parasites to drug sensitivity, we deleted the encoding genes in *P. berghei*. Drug sensitivity assays on these transporter knockout lines gave no phenotype with conventional anti-malarial compounds (unpublished results)[274]. Multiple factors potentially influenced this outcome, such as the short duration of the assays (24 hours), the quality of the parasites at inoculation and the improved development of *P. berghei* parasites in reticulocytes, which are scarce in peripheral blood samples used for culturing in these assays[274]. *P. falciparum* drug sensitivity assays were performed on the *mrp1* and *mrp2* gene deletion mutants, and similarly show no effect for the selected drugs, except for a small decrease in mefloquine sensitivity **(Chapter 4)**. Raj *et al.* did find an increase in sensitivity of MRP1-deficient mutants to chloroquine, quinine, artemisinin, piperaquine and primaquine, resulting in a decreased IC_{50} concentration in the chloroquine-resistant W2 strain[64]. We, however, deleted the MRP-encoding genes in a sensitive parasite background, NF54, and the generally low transport

capacity of endogenously expressed ABC transport proteins further reduced the chance of detection of differential sensitivity. Moreover, we removed the selectable marker though enhanced flippase (FLPe)-mediated recombination, whereas the deletion of *mrp1* by Raj *et al*. was exerted in a single crossover fashion, and heterologous DNA including the selectable marker was maintained. These discrepancies in the model organisms may (partially) explain the differential outcomes in antimalarial sensitivity.

Plasmodium ABC transporter substrate identification

The identification of specific *Plasmodium* ABC transporter substrates is of critical importance to explore their physiological roles in (fundamental) cellular processes, such as homeostasis and signaling. A recently described untargeted metabolomic approach by Jansen et al.[155], has been developed for the crude detection of differentially distributed physiological substrates in extracellular compartments of wild type versus *mrp*-deleted cells. In this way, differential export of metabolites can be detected in the extracellular matrix, a technique we applied on our *mrp*-deleted *P. falciparum* parasites as described in **Chapter 3**. We did indeed detect decreased folate concentrations in the red blood cell compartment of *PfΔmrp1* schizont-infected erythrocytes, which was confirmed with targeted liquid chromatography/tandem mass spectrometry (LC/MS-MS). As folate is a known substrate of human MRP transport proteins, it is likely that it is exported by *Pf*MRP1[192].

A role of MRP protein expression in folate homeostasis, rather than involvement in autocrine or signaling pathways, has been suggested in humans[166]. The conservation of *de novo* synthesis enzymes in *Plasmodium* species[167,168] underlines the high demand for folate during specific life cycle stages in which proliferation is unparalleled[169]. Although folate is involved in multiple processes including DNA translation initiation and methylation of DNA and proteins, its function as one-carbon donor in purine synthesis is most fundamental and explains its high consumption during cellular replication[170,171]. The extent to which folate is exported in other proliferative life cycle stages such as the oocyst- and liver stages, still needs to be explored. Furthermore, alternative substrates may be more abundant in these life cycle stages. However, obtaining large quantities of purified mosquito and liver stage parasites required for such metabolomic studies has proven to be a great challenge.

We observed a significantly reduced sensitivity of mrp1-deleted parasites for the antifolate methotrexate. Because of reduced export, folate concentrations are presumably increased, which

could have effectively antagonized the improved affinity of methotrexate for dihydrofolate reductase (DHFR), resulting in decreased methotrexate sensitivity. The beneficial effect of an increased folate concentration on survival of antifolate treatment has been observed previously[184,186]. However, the anti-folates pyrimethamine, WR99210 and trimethoprim did not show a differential effect. Antifolates inhibit folate synthesis at two levels; (1) during *de novo* synthesis, where the para-aminobenzoic acid (pABA) and hydroxymethyl dihydropteridine pyrophosphate components are joined into dihydrofolate by the hydroxymethyl dihydropteridine pyrophosphokinase – dihydropteroate synthase enzyme complex, or (2) during conversion of dihydrofolate to tetrahydrofolate by the DHFR enzyme. Sulfadoxine, sulfalene, sulfamethoxazole and dapsone are structural analogues of pABA. The sulfa-adducts that are incorporated with these compounds lead to inhibition of enzyme activity downstream in folate metabolism. DHFR is the target of a second set of anti-malarial antifolates: pyrimethamine, proguanil, methotrexate, trimethoprim and WR99210. These compounds in general bind to DHFR with increased affinity, thereby inhibiting its function. Methotrexate is the only structural analogue of folate, and the resistance towards this compound of *mrp1*-deleted parasites may therefore be explained by direct competition at the DHFR binding pocket.

Other more fundamental questions regarding the characteristics of substrate-transporter interaction, such as type of enzyme kinetics and the effect of amino acid substitutions on substrate specificity, cannot be answered using this metabolomics approach. Therefore, functional expression and direct measurement of vesicular transport of potential substrates in a heterologous overexpression system would be the method of choice, by using either radiolabelled compounds or LC/MS-MS detection[100,275]. Alternative options to identify substrates include the application of specific inhibitors and/or cellular accumulation assays to evaluate reduced translocation that can be attributed to the function of specific ABC transporters[276,277]. However, *Plasmodium* studies are faced with multiple obstacles. The selectivity of currently available inhibitors for ABC transport proteins is poor, while heterologous overexpression remains a technical challenge as *P. falciparum* DNA is extremely AT rich typically constituting 70-80% of the open reading frame. Mutation-free PCR amplification of the large ABC transporter coding genes[37] is another stumble block as codon usage is very atypical in *Plasmodium* species. This hampers effective translation in heterologous organisms where truncation has been observed[162]. Functional expression of *P. falciparum* MDR1 has been reported in *Pichia pastoris* and *Xenopus laevis* oocytes[41,43]. In our hands, however,

expression of *Pf*MRP1 and *Pf*MRP2 has remained unsuccessful[278]. Future attempts might work with larger availability of custom-designed DNA sequences in which codon-usage can be optimized or harmonized. Since heterologous overexpression failed in *E. coli*, *Xenopus laevis* oocytes, Sf9 insect cells and *Dyctiostelium discoideum*, expression in *Toxoplasma gondii* might be an attractive alternative. This apicomplexan parasite is closely related to *Plasmodium* species and shares distinct biological properties, such as the presence of an apicoplast as well as a high content of A and T bases in genomic DNA with distinct codon usage[279]. *Toxoplasma gondii* can be easily manipulated, which would aid the introduction of *P. falciparum* genes. Different types of yeast could be also tried for functional *Pf*ABC transporter expression, as these cells can sometimes be optimized for proteins that are difficult to express[280]. Endogenous overexpression in *Plasmodium* species could also be attempted through epigenetic expression or promoter swapping. Major disadvantages of this strategy are the difficulties in *Plasmodium* manipulation, especially when large constructs need to be transduced, and the large-scale cultures that need to be processed in order to obtain substantial parasite specific membrane fractions, as we experienced ourselves (unpublished results).

Essential role of MRP2 for hepatic development

While MRP transporters of the ABCC subfamily could be stably deleted in both *P. berghei* and *P. falciparum*, MRP2 appeared to be essential for liver-stage development, as described in **Chapter 4.** *Mrp2*-deficient parasites readily invade hepatocytes at WT rates and establish a parasitophorous vacuole (PV) as shown by the expression and correct localization of exported protein 1 (EXP1), but are unable to form mature liver schizonts as shown by the reduced size, annihilated nuclear multiplication and the absence of MSP1, a marker for merozoite formation[239]. In fact, the lack of *mrp2* alone results in complete developmental arrest of liver stages, while the phenotype of the *mrp1* gene deletion mutant does not alter in any of the *P. falciparum* life cycle stages. This indicates that MRP2 transports essential substrates during liver-stage development, a function that apparently cannot be compensated for by MRP1 or other (ABC) transporters. Rodent *Plasmodium* species encode only a single MRP protein, whereas in human parasites two members have been identified. The single copy *abcc* gene of *P. berghei* seems to be the syntenic ortholog of *Pfmrp2* as it shows a higher sequence similarity to *Pfmrp2*, suggestive for functional orthology of the *P. berghei* MRP protein and *Pf*MRP2. Indeed, upon the deletion of the *P. berghei mrp* gene, we observed a phenotype of aborted liver-stage development comparable to *Pfmrp2* deletion.

This important finding creates exciting opportunities for clinical development of drug leads specifically targeting pre-erythrocytic stages and/or whole sporozoite-based vaccines. As treatment of malaria infections is threatened by the emergence and spread of drug resistance including artemisinins[8,281], and with a renewed impulse for malaria elimination, measures preventing clinical episodes of malaria are urgently required. Interventions during liver stage infection are an elected opportunity to battle infection before symptoms arise; for instance by targeting the parasite using pharmaceuticals. The demand for effective liver-stage drugs is specifically high, because the only effective pre-erythrocytic drug that is currently available is primaquine[282]. This compound unfortunately causes hemolysis in individuals carrying glucose-6-phosphate dehydrogenase (G6PD) deficiency[283], common in malaria endemic regions, which is also compromising the effective treatment of hypnozoites and thus malaria relapse in *P. vivax*-infected individuals. As MRP2 is essential for hepatocytic-stage development of the parasite, targeting this protein or its substrates could be a valuable novel strategy.

Vaccination against pre-erythrocytic stages would be a powerful tool to control or even eliminate malaria. Subunit vaccination strategies have proven notoriously difficult. The only subunit vaccine candidate that is currently completing phase three of clinical trials, RTS,S[284], is based on circumsporozoite surface protein (CSP), and unfortunately does not show efficacy exceeding 36,3%, which is likely insufficient for elimination strategies[285]. Pre-erythrocytic stage immunizations with attenuated whole sporozoites have proven their superior potential[285], as sterile protection can be achieved using the different strategies. These include radiation-attenuated sporozoites (RAS)[286], or under cover of chemo-prophylaxis (CPS)[287]. However, for mass administration both strategies are suboptimal, as irradiated sporozoites represent a genetically diverse population and adherence and efficiency of prophylactic strategies in chemical attenuation is essential to prevent episodes of vaccine-induced malaria. Another strategy comprehends of the application of genetically attenuated sporozoites (GAS), which constitute of a genetically homogenous population without the need for application of pharmaceuticals to prevent breakthrough to blood stage infection[240].

A number of rodent GAS have previously been reported to arrest at early time points during development in the liver[240,242]. These include GAPs based on genes essential for the formation and maintenance of a PV (*b9, p52, p36, uis3* and *uis4*; [243-245]), type II fatty acid synthesis (i.e. *fabb/f, fabz, pdh e1a*;[246,247]), and regulation of gene expression (*sap1/slarp*[248-250]). However, many of these

deletion mutants show leaky phenotypes, resulting in blood stage infections. Incomplete liver stage arrest obviously disqualifies GAPs for further clinical development for safety reasons. The *PbΔmrp2* gene deletion mutant, however, showed no breakthrough to blood-stage infection upon injection with a high doses (300.000) sporozoites in C57bl/6 mice, the most sensitive mouse model. The only alternative GAP candidate that arrests completely during liver stage is lacking the sap1/slarp gene[3,249,250,252]. This GAP arrests early after hepatocyte invasion, as these parasites do not construct a functional PV[249,250]. Immunization with Slarp deleted parasites, however, does not result in consistent protection[250,288]. Moreover, there is evidence that GAP immunizations with a later arresting phenotype induce superior immune responses resulting in higher protection in mice, presumably through the prolonged presentation of a larger repertoire of antigens [253,254]. Parasites lacking MRP2 develop to a further extent compared to the safe and early arresting GAP, as nuclear division is observed with formation of a PV maintained and they persist minimally for the duration of a normal liver stage infection in hepatocytes. The presumed prolonged presentation of a larger repertoire of antigens, including PV-associated proteins such as EXP1, may be a benefit for efficient induction of protective immunity. In preliminary experiments, we have immunized BALB/c mice with low dose *PbΔmrp2* sporozoites, indeed resulting in protection against a WT challenge. Further experiments are needed in the more stringent C57BL/6 mouse model to characterize the protective potential of this GAP, including long-term protection. Comparing multiple GAP candidates should give a final verdict on the benefit of these later arresting sporozoites.

For the application of safe GAP vaccination strategies, it has been previously suggested that a minimum of two essential genes have to be deleted in order to ensure parasite stability and minimize the risk of reversion to a vital phenotype during liver stage development[215]. Vaccine candidates that are currently being evaluated for human administration, however, lack two essential genes that are involved in similar pathways of membrane biogenesis, resulting in absence of a PV during liver stage development. The deletion of two genes in unrelated pathways that play vital roles in parasite development during liver stage would be a more ideal safety measure, minimizing the chance that adaptation of the parasite could lead to an effective completion of the liver stage and consequent blood stage infection upon vaccination. As MRPs are a different class of proteins with functions in alternative cellular processes, a tandem deletion with mrp2 would be an attractive safety measure. So far, additional proteins known to play a vital role during the later stages of intra-hepatic parasite development are not identified yet, which for now restricts the opportunity to take advantage of *PfΔmrp2* as a late stage arrester.

***Plasmodium falciparum* liver stage assay**

Improvement of the liver stage assay for human *Plasmodium* parasites is of essential importance, as currently, in vitro options are scarce and notoriously result in very limited numbers of infected hepatocytes. Not only the production of large numbers of liver stage parasites could hold the key to the characterization of parasite biology during this stage, including the identification of MRP2 substrates and essential pathways they are involved in using metabolomics, but also sensitive assays are needed for detection of trace parasites that escape liver stage developmental arrest. Furthermore, studying *P. vivax* hynozoite biology for the development of drugs targeting these exo-erythrocytic forms (EEF) would stimulate another field that is currently under-represented.

The only immortalized cell line supporting liver-stage development for *P. falciparum* are HCO4, indeed resulting in very low infection rates[289]. Furthermore, fresh primary human hepatocytes have been used[290]. Sporozoite infection rates in these cultures (around 0,5-1% in our hands) are higher as compared to HCO4 cells (around 0,05% in our hands)[291]. The use of freshly isolated human material comes with significant disadvantages, as it is a labor-intense process, cells can be cultured for only approximately 14 days, the availability of human liver tissue is unpredictable, there is large variation in cell viability upon extraction and inter-donor differences in infection rates limits standardization of the assays.

Novel assays should ideally fulfill the following requirements; 1) High recovery and viability after cryopreservation. 2) Inducible high multiplication rates to produce sufficient cells in a short amount of time to accommodate all required experimental conditions. 3) High sporozoite infection rates. Expression of CD81 is the only marker that is currently associated with parasite invasion and can be evaluated[292], however, multiple factors are most likely involved in invasion and establishment of hepatocytic infection. 4) Parasites develop into fully matured schizonts. 5) Cells do not multiply during the infection with *P. falciparum*, as this would dilute the number of parasites.

Currently, many strategies for optimization of primary human hepatocyte cultures are being evaluated, such as efficient cryopreservation and co-culturing of micropatterned hypatocytes with supportive stromal cells, which contribute beneficially to the longevity of cryopreserved primary hepatocytes in vitro[291,293]. Furthermore, growing primary human hepatocytes under hypoxic conditions has shown some efficacy on the increase of parasite infectivity and support in hepatic-

stage development[294] and the use of extracellular matrix mimics, including matrigel and structured surfaces or flowcells are being explored. For development of a routinely used in vitro hepatic-stage assay, the use of either human hepatocyte stem cells or immortalizing primary hepatocytic (stem) cells that can either proliferate or differentiate in a static culture depending on temperature using both the temperature-sensitive mutant U19tsA58 of SV40 large T antigen (SV40T) and the essential catalytic subunit of human telomerase (hTERT) system[295,296] might be novel strategies holding potential of studying hepatocyte-related processes for many purposes.

Vital and dispensable roles of *Plasmodium* MDR transporters

In **Chapter 5**, we revealed another physiological role of ABC transporters in *P. berghei*. The majority of MDR encoding genes (*mdr1*, *mdr4*, *mdr6* and *mdr7*) could not be deleted and are apparently essential for blood-stage multiplication. *Mdr2*, *mdr3*, and *mdr5* have been successfully deleted in both *P. berghei* and *P. falciparum*. These three genes are transcribed in blood stages (www.plasmodb.org)[271], suggesting that they play a role during this developmental phase. Although the ability to delete these genes may indicate that other proteins compensate for the loss of MDR transporter function, the reduced growth we observed for *P. berghei* blood stages lacking expression of MDR5 strongly suggest a role in these stages. However, and in contrast to the *P. berghei* results, we did not observe a decreased growth rate for the *Pfmdr3* and *Pfmdr5* gene deletion mutants, whereas maximal parasitaemia was inhibited in *Pfmdr2* deletion mutants. This may indicate that the function of the orthologous MDR proteins differs between blood stages of *P. berghei* and *P. falciparum* or that in *P. falciparum* the loss of these MDR proteins can be compensated by other proteins. However, these discrepancies may also be due to different assays used for analysis of blood stage growth. For *P. berghei*, blood stage growth was determined *in vivo*, whereas *P. falciparum* growth was analyzed under *in vitro* conditions.

Mdr2 and *mdr5* knockout parasites showed compromised formation of oocysts and maturation of sporozoites in *P. berghei* and strongly reduced oocyst and sporozoite numbers in *P. falciparum*. The effect of *mdr2* or *mdr5* deletion on *P. berghei* maturation was most clearly visualized in the parasite mutants where only fully mature oocysts express GFP, as a strong and significant reduction in GFP-positive oocysts was observed. In both *P. berghei* and *P. falciparum*, the absence of MDR2 and MDR5 did not result in a complete block of sporozoite formation. These proteins apparently play a role, although non-essential, in oocyst maturation, and more detailed investigation of the substrates and

pathways that are involved in this mechanism may provide valuable information on oocyst biology and opportunities to interfere with parasite development during transmission stages.

Options for *P. falciparum* gene manipulation

ABC transport proteins play an important role in maintaining homeostasis in many organisms, and could be of special importance for parasites interacting with their host cell environment from which they require essential nutrients and to where they dispose of toxic waste products. For example, MDR transport proteins are known to play a role in lipid metabolism and phosphatidylcholine transport in many organisms, and as membrane biogenesis peaks during sporozoite formation this might be one of the factors contributing to the observed phenotype in mosquito stage development[297]. Elucidation of the vital role of these transporters in different stages of the parasite life cycle may reveal novel drug targets for inhibition of parasite development and transmission prevention.

P. berghei mdr1, *4, 6, 7*, and *P. falciparum mdr6* are essential for asexual multiplication, however, we have not been able to study ABC transporter genes that are essential during blood stage as parasite transfection and selection is executed in these stages. From a biological and drug development perspective, these genes are of particular interest. Specific targeting of transport function of these *Plasmodium* ABC proteins could potentially lead to a novel group of anti-malarial compounds. For that purpose, expression regulation, conditional deletion and the introduction of single nucleotide mutations will be essential. Molecular techniques that can be applied to study gene function in other (model) organisms are unfortunately not available in *P. falciparum*. One example is RNA interference (RNAi), where short inhibitory RNA (siRNA) or short hairpin RNA (shRNA) is transfected into cells and accounts for mRNA degradation and consequently downregulation of protein expression, abusing cellular antiviral defense mechanims[298]. Because the encoding genes for the RNAi machinery are not present in *Plasmodium* species, manipulation at this level is not possible[299].

P. berghei parasites can be transformed during merozoite stage, because under these conditions only the parasite and nucleic membrane have to be crossed to target DNA. This higher transfection efficiency enables direct selection of double crossover parasites, into which linearized targeting constructs are stably integrated[204]. In *P. falciparum*, this process is much less efficient as early ring stage parasites can only incorporate circular DNA constructs upon electroporation, and this

DNA has to pass four membrane barriers; the erythrocyte, parasitophorous vacuole, plasma, and nucleotide membrane[211,300]. Furthermore, transfection efficiency declines with increasing construct size, implying difficulties in the introduction of large genes, such as the >6kB constructs encoding ABC transporter proteins[204].

Despite of these drawbacks, remarkable progression in molecular manipulation of *P. falciparum* parasites has been made in recent years. Successful application of the FLP-FRT and the Cre-loxP recombination systems has been reported upon optimization for *P. falciparum*[152,301]. These systems enable the excision of DNA sequences intermediary of two FRT or loxP sites, a technique which we also used for the removal of the positive selection marker and the subsequent deletion of a second *mrp* gene in order to delete the entire MRP family in *P. falciparum*. Besides the possibility to remove multiple genes with this technique, it has also been used for conditional deletions in many organisms by placing Cre or FLP encoding genes under control of a differentially active promoter[302]. This application has also been reported in *P. berghei*, where conditional deletion of genes was achieved by placing FLPe expression under control of a sporozoite-specific promotor[241,303-305]. However, for application in *P. falciparum*, the impossibility of introducing linearized DNA is interfering with correct integration of the FRT sites as well as practical feasibility due to prolonged culturing requirements. Conditional deletion of loxP-flanked sites was achieved in *P. falciparum* using a DiCre system, where Cre recombinase is expressed in two separate units containing rapamycin binding domains that combine and become functional upon the addition of rapamycin to the culture[306,307]. Again, the introduction of the loxP sites at the correct positions in the genome is challenging and time-consuming in *P. falciparum*.

Another relatively new application in *P. falciparum* forward genetics, aiming at the identification of genes involved in specific phenotypes, is the piggyBac transposon system. Insertion of this transposon resulted in random integration into the parasite genome, however, integration into *P. berghei* genomic DNA was more efficient, most likely due to the higher transfection efficiency[308,309]. Furthermore, tagging with a destabilization domain (DD) has been attempted with some success, in order to target the protein for proteosomal degradation upon withdrawal of stabilizing compounds[310-313]. However, the DD tag should not affect protein function and trafficking towards the proteasome should not be compromised in order to achieve efficient knockdown. Recently, another type of post-translational regulation in the form of an auxin-based degron system has

been successfully applied in *P. falciparum*[314]. Although the advantage of this system is that protein expression can be reversed, the applicability for all targets and the effect on protein function still needs to be investigated.

A leap forward in genome editing of *P. falciparum* was achieved in 2012 through the successful application of zinc finger nucleases for site-specific rearrangement of genomic DNA[315]. As zinc fingers are targeted against specific sequences and introduce double strand breaks at this site, insertion of large homologous regions for plasmid integration is not required. There are, however, limitations concerning the availability for all required sequences and the specificity for the excision site, as well as the time-consuming and expensive commercial design of sufficiently specific zinc finger combinations. These drawbacks are reflected in the lack of publications yet on applying zinc finger technology for genome editing.

The successful application of a novel and exciting genome editing technique, the CRISPR/Cas9 system, has been reported in *P. falciparum*[316,317]. In this system, the Cas9 enzyme is guided to the specific DNA strand using a single guide RNA, after which a double strand break is introduced. Template DNA is presented for double strand break repair, by which mutations can be introduced, and genes deleted or inserted. Major advantages of this technique are that it only requires the introduction of DNA plasmids and even linearized DNA was shown to be integrated, possibly due to the high integration efficiency.

Applying these novel techniques to study the function of essential ABC proteins could be of help in identifying their contribution to specific genotypes more rapidly through forward genetics. Especially conditional gene deletion holds the potential of identifying the exact erythrocytic stage at which these genes are essential and evaluate the effect of their absence in mosquito- and liver-stage parasites. Furthermore, the application of CRISPR/Cas9 technology can contribute to our knowledge of the effect of single base pair substitutions in ABC proteins on parasite drug sensitivity and thus transporter characteristics. As it is clear from deletion experiments that many ABC transporter genes are essential during different life cycle stages, applying these techniques to identify their substrates and physiological functions could aid in unraveling parasite biology and revealing specific vulnerabilities.

Conclusion

The association of ABC transporters with drug sensitivity has been the main focus of research in *Plasmodium* species during previous years **(Chapter 1)**. Whereas single nucleotide polymorphisms and variation in expression had been associated with altered drug sensitivity of the parasite, limited functional data were available. This thesis has given a first indication of substrates under physiological conditions and has underlined the vital roles of ABC transport proteins in *Plasmodium* parasite development during different life cycle stages. Next steps would be to elucidate additional specific substrates and identifying pathways in which these transport proteins are involved in different life cycle stages through over-expression and genome editing by applying novel techniques for conditional gene deletion and mutagenesis. Exploitation of this knowledge, either by drug targeting of asexuals, transmission blocking opportunities, or by using gene-deleted parasites in hepatic stage vaccine strategies, might provide an alternative contribution to the toolbox for malaria eradication.

CHAPTER 7

Epilogue

Epilogue

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SUMMARY

Malaria tropica remains one of the largest threats to global health, as it results in a fatal outcome in approximately 600.000 cases annually. Especially children under five in countries with limited resources in sub-Sahara Africa are affected by the causative parasite, *Plasmodium falciparum*. An effective vaccine is not available despite of the many challenging research initiatives, and moreover, effective treatment is threatened by the emergence and spread of parasite resistance against the first-line drug class of artemisinins. ATP-binding cassette (ABC) proteins are known for their involvement in multidrug resistance in many organisms, as these transporters located at membranes can export a wide variety of compounds including drugs away from their target site. Also in *Plasmodium falciparum*, single nucleotide polymorphisms, genomic duplication or altered expression of these transporters has been associated with decreased drug resistance.

In **Chapter 1** we described the general structure of all ABC transporters in *Plasmodium* species, including a phylogenetic analysis. Furthermore, we provided an overview of the current knowledge on the association of specific mutations and expression variation of *Plasmodium* ABC transport with antimalarial sensitivity.

Next, we explored the interaction of commonly used antimalarial compounds with human ABC transport proteins in **Chapter 2**. Vesicular transporter overexpression assays identified a strong inhibitory effect of quinine and mefloquine on P-glycoptorein-mediated transport and of mefloquine, atovaquone and proguanil on that of breast cancer resistance protein (BCRP); indicating that these compounds may be involved in drug-drug interaction with substrates of these transporters.

In **Chapter 3** we set out to identify physiological substrates of MRP1 and MRP2 transporters, which were stably deleted from the *P. falciparum* genome through double crossover recombination. Using an untargeted metabolomic approach and applying LC-MS/MS analysis, we identified a decreased concentration of folate in the red blood cell compartment of *PfΔmrp1* schizont-infected erythrocytes. Furthermore, we performed drug sensitivity assays for a number of antifolate compounds, and observed a significantly reduced sensitivity of *mrp1*-deleted parasites for the antifolate methotrexate. Both results and the fact that folate is a known substrate of human MRP transport proteins suggest that indeed folate is exported by *Pf*MRP1.

Phenotype analysis of *mrp*-deleted parasites in different life cycle stages of *P. berghei* and *P. falciparum* was described in **Chapter 4**. Although these parasites developed normally during blood- and mosquito stages, MRP2 had an essential function during liver stage maturation. A fluorescently tagged MRP2 orthologue in *P. berghei* shows localization of the protein at the plasma lemma of the liver-stage parasite, which is confirmed for both MRP1 and MRP2 using immunofluorescence in *P. falciparum*. For both species, hepatocyte traversal and invasion of *mrp2*-deleted parasites was not affected, and also the construction of a parasitophorous vacuole was successful, as shown by the presence of EXP1 expression. However, nuclear multiplication was significantly inhibited, and parasite size and survival rates are reduced most strongly in *P. falciparum*. Furthermore, expression of MSP1, a marker for liver stage maturation, is not present in both species. *Mrp2*-deleted sporozoites could not induce blood stage infection; indicating complete liver stage attenuation. This represents an important novel finding, stressing the essential functions of ABC transporters in *Plasmodium* species, which can be exploited in pre-erythrocytic drug development and/or genetically attenuated whole sporozoite vaccination strategies.

In **Chapter 5** we targeted the complete multidrug resistance (MDR) ABC transporter subfamily in *P. berghei*, and successfully deleted *mdr2*, *mdr3* and *mdr5*. Despite of multiple attempts, parasites lacking expression of MDR1, MDR4, MDR6 and MDR7 could not be obtained, indicating that these proteins have essential functions during blood stage development. These results were confirmed in *P. falciparum* as attempts to delete *mdr6* were unsuccessful, whereas *mdr2*, *mdr3* and *mdr5* could be removed. Phenotypic evaluation of mdr deleted parasites showed a reduced blood stage multiplication rate of *PbΔmdr5* and a reduced maximal parasitemia for *PfΔmdr2*. More strikingly, in both *P. berghei* and *P. falciparum*, development of *mdr2* and *mdr5* deleted parasites during mosquito stages resulted in decreased numbers of oocysts and sporozoites. Evaluation of mdr-deletion in *P. berghei* parasites expressing GFP only in mature oocysts showed a specific effect on this stage.

The combined data showed that ABC transport proteins may play important roles in *Plasmodium* drug interactions and sensitivity, and have both essential and otherwise important functions during different parasite stages. Identification of specific substrates of these transporters and further exploration of the pathways will aid in targeting these parasites at different key point of their life cycle.

SAMENVATTING

Malaria tropica blijft één van de grootste bedreigingen van de wereldgezondheid, aangezien het jaarlijks tot ongeveer 600.000 sterfgevallen leidt. Vooral kinderen jonger dan vijf jaar in de arme landen ten zuiden van de Sahara zijn het slachtoffer van *Plasmodium falciparum*, de parasiet die deze ziekte veroorzaakt. Ondanks jarenlang onderzoek is een zeer effectief vaccin nog niet beschikbaar, en daarbij wordt de huidige behandeling met artemisinine in combinatie met andere antimalariamiddelen bedreigd doordat resistente parasieten zijn opgedoken en zich verspreiden. ATP-binding-cassette (ABC)-eiwitten staan bekend om hun aandeel in het veroorzaken van resistentie tegen een groot aantal geneesmiddelen in verschillende organismen. Het zijn membraantransporteiwitten die een breed scala aan stoffen actief uit de cel kunnen pompen en daarmee wegtransporteren van hun aangrijpingspunten. In *Plasmodium falciparum* spelen mutaties of verhoogde expressie van deze transporters een rol bij een verlaagde gevoeligheid voor antimalariamiddelen.

In **Hoofdstuk 1** hebben we de structuur van ABC-transporters die in het genoom van *Plasmodium*-parasieten gecodeerd zijn beschreven, inclusief een phylogenetische analyse. Wij hebben een samenvatting gegeven van de huidige kennis op het gebied van specifieke mutaties en variatie in expressie van *Plasmodium* ABC-transporters en hoe dit in verband staat met de gevoeligheid van de parasiet voor antimalariamiddelen.

Vervolgens hebben we in **Hoofdstuk 2** de interactie onderzocht van veelgebruikte antimalariamiddelen met humane ABC-transporters. Experimenten met membraanvesicles waarin transporter-eiwitten tot over-expressie zijn gebracht lieten een sterk remmend effect zien van quinine en mefloquine op de transportactiviteit van P-glycoproteine, alsmede een vergelijkbaar effect van mefloquine, atovaquone en proguanil op transport door breast-cancer-resistance-protein (BCRP). De resultaten suggeerden dat deze antimalariamiddelen betrokken kunnen zijn bij interacties met andere geneesmiddelen die substraten zijn van deze transporters.

In **Hoofdstuk 3** hebben we getracht de fysiologische substraten te identificeren van multidrug-resistance-associated-protein MRP1- en MRP2-transporters na genetische verwijdering uit het genoom van *P. falciparum*. In een 'non targeted'-metabolomicsstudie en LC-MS/MS-analyse hebben wij een verlaagde concentratie van folaat gevonden in het cytosol van erythrocyten geïnfecteerd door

schizonten met een *Pfmrp1*-deletie. Verder hebben we in sensitiviteitstesten voor een aantal antifolaten aangetoond dat de gevoeligheid van parasieten met een mrp1-deletie voor methotrexaat significant is afgenomen. Gezien het feit dat folaat een bekend substraat van humane MRP-transporters is, suggereerden de gecombineerde resultaten dat folaat getransporteerd wordt door *Pf*MRP1.
Het fenotype van parasieten met een *mrp*-deletie is in **Hoofdstuk 4** geëvalueerd in verschillende stadia van de levenscyclus van zowel *P. berghei* als *P. falciparum*. Hoewel beide parasieten zich gedurende de bloed- en muggenstadia normaal ontwikkelen, bleek MRP2 een essentiële functie te hebben gedurende de rijping in het leverstadium. De ortholoog van MRP2 in *P. berghei* lokaliseerde op de plasmamembraan gedurende het leverstadium wat ook geldt voor zowel MRP1 als MRP2 in *P. falciparum*. In beide speciës was de hepatocyt-passage en -invasie van parasieten met een *mrp2*-deletie niet aangedaan, en bleek ook de vorming van de parasitofore vacuole onveranderd. De nucleaire multiplicatie was echter wel sterk verminderd en ook overleving en groei van met name de *P. falciparum*-parasieten is sterk gereduceerd. Expressie van MSP1, een marker voor uitrijping in het leverstadium, was in beide speciës afwezig. *P. berghei- mrp2*-deletie mutanten gaven geen bloedstadium infectie waarmee voor het eerst een essentiële rol van ABC-transporters in *Plasmodium* werd aangetoond. Dit biedt mogelijkheden voor ontwikkeling van vaccins en nieuwe geneesmiddelen.

In **Hoofdstuk 5** hebben we geprobeerd alle genen van de multidrug-resistance (MDR) ABC-transportersubfamilie te verwijderen uit het genoom van *P. berghei*, wat is gelukt voor *mdr2*, *mdr3* en *mdr5*, maar niet voor *mdr1*, *mdr4*, *mdr6* en *mdr7*. Dit wijst erop dat deze eiwitten waarschijnlijk een essentiële functie hebben gedurende de ontwikkeling van de parasiet in het bloedstadium. Vergelijkbare resultaten werden verkregen in *P. falciparum*, waarin het ook onmogelijk is gebleken *mdr6* te verwijderen, terwijl parasieten met een *mdr2-*, *mdr3-* en *mdr5*-deletie wel geproduceerd konden worden. Fenotypische analysen van parasieten met een deletie van één van deze genen lieten zien dat er een verminderde groei gedurende het bloedstadium was voor *P. berghei*-parasieten zonder expressie van MDR5, en dat de maximale parasitemie lager was voor *P. falciparum*-parasieten zonder MDR2-expressie. Nog opvallender was dat in zowel *P. berghei* en *P. falciparum* de ontwikkeling van parasieten met een *mdr2-* of *mdr5*-deletie gedurende het muggenstadium resulteerde in verminderde aantallen oöcysten en sporozoïten. Nadere bestudering van *P. berghei* parasieten met een *mdr*-deletie waarbij alleen volledig uitgerijpte oöcysten een fluorescent signaal gaven, liet zien dat specifiek deze uitrijping was verstoord.

Concluderend laten deze resultaten een belangrijke rol van humane ABC-transporters zien bij geneesmiddeleninteracties met antimalariamiddelen. Voorts vervullen deze transporters essentiële functies gedurende de verschillende ontwikkelingsstadia van de parasiet. De identificatie van specifieke endogene substraten van deze transporters en verder onderzoek naar de biologische mechanismen bieden nieuwe mogelijkheden om de parasiet te bestrijden gedurende belangrijke fases in de levenscyclus.

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CURRICULUM VITAE

Sanna Rijpma was born in 1985 in Dunedin, New-Zealand, as the daughter of a Frisian father and Dutch mother. After almost four years, her family moved back to the Netherlands and she spent her youth with her two younger sisters in Silvolde. There she went to the Almende College, Isala site high school, where she was educated in the Nature and Technical as well as the Nature and Health profile, with additional subjects economy and geography. She graduated *cum laude*, and spent a semester at the RijksUniversiteit Groningen, where she studied international relations and international organizations. Then, she started her bachelor biomedical sciences at the Radboud University Nijmegen, during which she was a representative for monitoring quality of education, and did an internship at the department of Pharmacology and Toxicology, entitled "Cloning and expression of ABC-transporters from *Plasmodium falciparum*". For this work, she was awarded the Dr. J. Bex prize in 2007 for best bachelor's thesis in biomedical sciences. During her master's studies she completed a research profile in toxicology and pathobiology and did an internship at the RIVM National Institute for Public Health and the Environment in the Laboratory of Health Protection Research on "Reproductive toxicity of Selective Serotonin Reuptake Inhibitors antidepressants in the Embryonic Stem cell Test". Furthermore, she did an internship at the NKI, the Dutch Cancer Institute, at the Division of Molecular Biology, entitled "Characterization of cell cycle checkpoints in cisplatin-surviving *Brca-1*; *p53*-deficient mouse mammary cancer cells", for which she was awarded the Faculty prize of the Radboud University for best master's thesis in biomedical sciences. She obtained an UMC St. Radboud personal grant for her PhD project, which she set out to do on ABC transport proteins in *Plasmodium falciparum*, resulting in this dissertation. Currently, she is working at the department of Medical Microbiology at the Radboudumc in the group of Teun Bousema on molecular and immonological mechanisms of malaria transmission.

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Sanna